ATTORNEY DOCKET: 46309-257438

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Robert David Possee et al.	)	
	)	Examiner: **Marvich, Maria**
Serial No. **09/807,809**	)	
(National Phase of PCT/GB00/03114)	)	Art Unit: **1636**
	)	
Filed: **July 30, 2001**	)	
	)	
For: **BACULOVIRUS EXPRESSION SYSTEM**	)	

DECLARATION OF ROBERT DAVID POSSE, PH.D. UNDER 37 C.F.R. §1.132

I, Robert David Possee, currently residing at 64 Millwood End, Long Hanborough, Witney, Oxon, OX298BY UK, do hereby declare:

1. I am an expert in the field of the invention. I am currently a researcher at NERC CEH Oxford (formerly Institute of Virology and Environmental Microbiology/Institute of Virology). I earned a B.Sc. degree in Biological Sciences with Honours in 1978 at the University of Birmingham, U.K. I earned a Ph.D. degree in virology at 1981 at University of Warwick, U.K. My *curriculum vitae* is enclosed (Exhibit A). I published over one hundred papers and books in the field of biology. The list of the publications is enclosed (Exhibit B). I am a co-author, with Linda King, who is a named inventor of the U.S. Patent Application Serial No. 09/807,809, of a text book on baculovirus expression systems entitled "*The Baculovirus Expression System; a Laboratory Guide*" (1992), first ed., Chapman and Hall Publishers, London, UK (hereinafter referred to as "my text book"), which was submitted to

Publishers, London, UK (hereinafter referred to as "my text book"), which was submitted to the U.S. Patent and Trademark Office with the Information Disclosure Statement filed April 18, 2003. I am a named inventor of U.S. Patent Application 09/807,809 (hereinafter referred to as "the present application"), entitled "Baculovirus Expression System." I am familiar with the application and the Office Action mailed by the United States Patent and Trademark Office on August 26, 2004.

3. The term "a functional gene" recited in Claim 29 would be understood by a person of ordinary skill in the art in the field of virology. The term means a gene capable of performing its normal function. Thus, in the context of Claims 29-30, the term "a functional gene" means a gene required for viral replication. Moreover, the specification defines the meaning of the term "a functional gene" on page 6, lines 19-20.

4. The term "a functional gene necessary for restoring a functional gene" recited in Claim 29 would be understood by a person of ordinary skill in the art in the field of virology. The term means that the rescue vector encodes a gene product that replaces the non-functioning gene product. The specification defines the meaning of the term "a functional gene necessary for restoring a functional gene" on page 6, lines 16-17.

5. The term "functional fragments or mutations thereof" recited in Claims 31-32 would be understood by a person of ordinary skill in the art in the field of virology. One of ordinary skill in the art would know that variations of genes in general occur naturally or could be introduced in the laboratory without undue difficulty. For example, one of ordinary skill in

the art would know that the genetic code is degenerate and results in different codons encoding the same amino acid. Hence, one of ordinary skill in the art would know that different mutations can produce genes that are still functional and retain at least a part of their activity. Furthermore, even if a change in the nucleotide sequence of a gene results in a change in amino acid, this does not necessarily result in a change in activity of the gene product.

One of ordinary skill in the art would also know that it is possible to delete parts of genes to produce fragments whilst still retaining the activity of that gene. Experiments to identify mutants and create functional fragments are well within the capabilities of one of ordinary skill in the art, such as an average graduate student in the field of virology. It was demonstrated and disclosed in the present application that deleting part of a gene recited in Claim 31, *ORF 1629,* results in its functional fragment. The genes recited in Claims 31-32 were known to one of ordinary skill in the art before the priority date of the present application, and one of ordinary skill in the art would know what their functional fragments or mutations mean. Furthermore, as discussed in more detail below, there are other baculovirus genes from different baculoviruses that, while essentially similar to the recited genes, retain their function despite being smaller in size or having a slightly different nucleotide sequence.

6. One of ordinary skill in the art would know that the term "naked" recited in Claim 27 in reference to a baculovirus vector is implicitly supported by the specification. One of ordinary skill in the art would understand "naked" to mean that the baculovirus vector is

without binding proteins (in the case of virus DNA, without the protective particles). The publications are enclosed (Exhibits C and D) showing that the term "naked" is used by those of ordinary skill in the art in reference to nucleic acids that are at least partially devoid of the proteins that usually accompany those nucleic acids. Baculovirus vectors are conventionally used packaged within a particle. In contrast, the claimed baculovirus vector is used without a viral particle. It is naked, or without at least a part of its coat of viral protein.

The specification provides support for a baculovirus vector DNA that is naked, or without the viral proteins. On page 6, paragraph 3, the specification shows that the baculovirus nucleic acid is DNA. On page 15 paragraph 2, the specification shows that the DNA is prepared by extracting it from yeast, not from a viral particle. The DNA is purified on a sucrose gradient and is ethanol precipitated. These methods produce naked DNA. DNA within the viral particle cannot be made in yeast. Using naked DNA requires using lipofectin (see p. 17, bottom) in order to transfect the naked DNA into cells. If the DNA was not naked, lipofectin would not be needed. The co-transfection described on page 33, final paragraph, also uses the naked baculovirus vector. Thus, based on the specification, one of ordinary skill in the art would know that naked baculovirus vector is used, and that the term "naked" is implicitly supported by the specification.

7. One of ordinary skill in the art would know that the specification describes the functional genes recited in Claims 31-32 (*lef 1-12, dnapol, pl43, p35, ie-1-2, p47, ORF 1629* and *pp 31*) in such a way as to reasonably convey to one skilled in the art in the field of

virology that the inventors, at the time the application was filed, had possession of the claimed invention. The specification discloses the claimed method with three different variations of the AcMNPV genome. At the priority date of the present application, the genes recited in Claims 31-32 were all known to be involved in baculovirus replication, as stated in the specification on page 6, lines 8-16, and in my textbook on pages 8-13.

One of ordinary skill in the art would know how to use the genes recited in Claims 31-32. As noted above, those genes were known at the priority date of the application, and the application provides the references and clear instructions on how to create the claimed components. The teachings given in the application could be readily applied by a person of ordinary skill in the art, such as an average postgraduate student, to produce replication-deficient baculovirus vectors lacking the genes recited in Claims 31-32. Even undergraduate students working in my laboratory as a part of their training undertake similar work to manipulate virus particles. Furthermore, undergraduate students whom I teach also work on insect genomes in organised laboratory practicals as a part of their normal course work.

8. The term "functional fragments or mutations thereof" recited in Claim 31, is described in the specification in such a way as to reasonably convey to one of ordinary skill in the art that the inventors, at the time the application was filed, had possession of the claimed invention. The genes recited in Claims 31-32 were known at the priority date of the present application. It was also known at the priority date that variations of genes in general occurred naturally or could be introduced artificially without undue difficulty. For example, it is known

that the genetic code is degenerate and results in different codons encoding the same amino acid. Hence, it is known that different mutations can produce genes that are still functional and retain at least a part of their activity.

Furthermore, even if a change in the nucleotide sequence of a gene results in a change in amino acid, this does not necessarily result in a change in activity of the gene product. It is also known that it is possible to delete parts of genes to produce fragments whilst still retaining the activity of those genes. Indeed, experiments to identify mutants and create functional fragments are well within the capabilities of an average graduate student in the field of virology. For example, it was demonstrated and disclosed in the present application that deleting part of a gene recited in Claim 31, *ORF 1629,* results in its functional fragment. As discussed in more detail below, there are other baculovirus genes from different baculoviruses that, while essentially similar to the recited genes, retain their function despite being smaller in size or having a slightly different nucleotide sequence.

There is a large number of different baculoviruses (as acknowledged on pages 1 and 2 of my textbook) that all have common structure. They have a large, double-stranded, covalently-closed, circular genome of between 88 and 200 kbp. This is associated with a highly basic (arginine-rich) protein of 6.5 kDa within a rod-shaped nucleocapsid containing a 39 kDa capsid protein. The length of the capsid varies with genome size (200-400 nm) but the width remains constant at about 36 nm. The packaging of the nucleocapsids within lipoprotein envelopes and the variation of the number of nucleocapsids within each virus were

studied. These studies resulted in baculoviruses being classified into different groups, all of which were known at the time of the priority date of the present application, and are discussed on pages 1-3 of my textbook.

AcMNPV was used as an example in the present application because it is a virus that was previously used and studied at a molecular level. However, other baculoviruses were also found and characterised. For example, almost identical baculoviruses were found in *Trichoplusia ni*, *Galleria mellonella* and *Rachiplusia ou*. Efficient expression vectors were produced for *Bombyx mori*, (Bm) NPV. Indeed at the time of filing, the complete genomes of five baculoviruses had been completely sequenced. One of ordinary skill in the art would be able to apply the techniques disclosed in the current application to these and other baculoviruses. One of ordinary skill in the art would be able to identify those genes present in the baculovirus and adapt the teaching of the current application appropriately.

A paper published in 1998, which was submitted to the U.S. Patent and Trademark Office with the Information Disclosure Statement filed April 18, 2003, outlines that nineteen baculovirus genes are essential for late gene expression (Rapp, J.C., Wilson, J.A and Miller, L.A. (1998). Nineteen baculovirus open reading frames, including LEF-12, support late gene expression. *Journal of Virology* v. 72, pp. 10197-10206). The publication demonstrates that removing one of these genes from a mixture used to transfect insect cells abolishes the activity of a reporter gene held in another plasmid. Since late gene expression depends on this group of genes, removing any one of these genes from a baculovirus genome will be enough to

render the virus unable to replicate. Thus, one of ordinary skill in the art would know that the genes recited in Claim 31 are functional genes necessary for viral replication.

As noted above, at the time of filing of the present application, there were five entirely sequenced baculoviruses, all of which had some or all of the genes recited in Claim 31. Conservation of sequences between baculoviruses is such that one of ordinary skill in the art would easily recognize genes with the same function. Many of the genes recited in Claims 31-32 are known to be ubiquitous in baculoviruses. For example, *lef-2, lef-8, p143, ie-1* and *pp31* are found in all baculoviruses sequenced to date.

9. The invention as claimed in Claims 27-34 is novel and non-obvious over the publications cited in the Office Action mailed August 26, 2004. The claimed invention has a number of unexpected advantages over previously known uses of baculovirus vectors, such as the advantages detailed below.

I. The claimed method allows for production of a pure population of recombinant baculovirus not contaminated with the parental baculovirus, or the baculovirus without insertions of foreign DNA. This considerably reduces the number of passages through cells that are generally required to remove the parental contamination. The effect of having to spend time to pick recombinant stocks to remove this contamination is disclosed in the application, for example, in the table on page 4, for conventional techniques used in linear DNA. Thus, the present invention avoids parental virus contamination and saves considerable time and money in the laboratory.

II. The use of the intermediate host allows the production of large quantities of baculovirus DNA, which has the defective virus gene and so cannot replicate alone when transfected into insect cells. It only replicates in insect cells if a transfer vector with a foreign gene and a functioning copy of the defective gene is transfected into the insect cell so as to restore the baculovirus genome. It is not possible to produce this defective baculovirus DNA in insect cells because of the defect in the baculovirus genome.

III. The intermediate host allows the baculovirus to be easily maintained and to be amplified to give large amounts of virus DNA.

IV. Using naked purified viral DNA that is not within viral particles allows the material to be stably stored, for example, in a refrigerator. Viral stocks contained within viral capsids suffer from problems of low infectivity when stored. In my laboratory, we have naked baculovirus DNA that is still stable after ten years of storage.

V. Using naked DNA allows the baculovirus vector and foreign DNA to be co-transfected, rather than having a two-step system where the virus is infected, and then foreign DNA is transfected in a separate step. This saves time and money.

VI. The separate introduction of virus particles and plasmid DNA of previous methods is not considered by those of ordinary skill in the art to be an efficient process for the production of recombinants. DNA introduced by a viral particle will begin

replication. Unless the incorporation of the virus particles and the plasmid DNA is timed correctly, there can be zero production of recombinant viruses. The claimed method overcomes this problem

10. Clark *et al.* (hereinafter *Clark*) fails to teach, suggest, or provide motivation to derive the claimed invention and fails to render it obvious. *Clark* suggests, but does not provide an example of a recombinant baculovirus expression system not capable of being maintained in an intermediate host. *Clark* suggests the use of a modified baculovirus which lacks a functional p35 gene (see, for example, Figure 2). The product of the p35 gene allows the virus to replicate in Sf insect cells by preventing the cells from undergoing apoptosis. In the absence of p35 in the *Clark* vectors, Sf cells undergo apoptosis and the virus cell cannot complete its replication cycle. The only way to replicate the suggested vectors of *Clark* is to use a cell line where p35 is not needed to prevent apoptosis from occurring. The usual cells are *Trichoplusia ni.* (*T. ni.*), e.g. TN368 cells, which allow the mutated baculovirus to be successfully replicated prior to use.

Hence, the method suggested in *Clark* suffers from a serious flaw. In order to replicate the suggested virus one must use a host cell, *T. ni.*, which permits the baculovirus to replicate in sufficiently high numbers to allow for production of a stock of baculovirus for use as an expression vector. In doing so, one produces mutants. That is, one has a heterogeneous population of baculovirus vectors even before any experimentation is carried out on them. *Clark* uses TN368 cells to amplify their deficient virus. However, there is extensive literature

detailing the production of virus mutants in this cell line that are less efficient in the expression of very late genes than viruses produced in other cell lines. As the baculovirus expression system depends on the use of very late gene promoters to drive foreign gene expression, production of the desired protein will be reduced.

Another disadvantage of *Clark* method is that the virus can still replicate at low levels in the normal insect cells. In contrast, the method of the present application provides for substantially zero replication in normal insect cells. Generally, when baculovirus is used, there is some contaminating parental virus without foreign genetic material integrated into it. The relative levels of this parental virus can be reduced by, for example, passaging the virus several times through Sf 9 host cells, but this requires additional effort, and can take several weeks longer than the applicants' invention, which does not require such steps. This disadvantage is discussed in the specification in the paragraph bridging pages 2 and 3 of the application as filed. The method in *Clark* will produce baculovirus with mutations, and will also produce baculovirus vectors containing foreign gene inserts contaminated by parental baculovirus without those inserts.

Clark suggests the use of <u>only</u> SF9 insect cells, but fails to teach the use of any insect cells other than SF9. One of ordinary skill in the art would not be motivated to derive applicants' invention from the teachings of *Clark*. *Clark* states that his technique works, and fails to provide the motivation to use anything other than the identified system, which apparently is the best way of carrying out the *Clark* method. With the benefit of <u>hindsight</u> one

might consider changing the cells, but *Clark* fails to suggest or provide motivation for such a change. Although alternative insect cells could be used for propagating the defective virus and for making recombinant viruses, *Clark* fails to teach, suggest, or provide motivation for other uses of the alternative insect cells. *Clark* also teaches a defective parental virus that can still replicate/amplify, albeit inefficiently, in the Sf9 cell type. The defective virus of the present application cannot replicate/amplify in Sf9 or any other insect cell type without the restoration of, for example, the *ORF 1629* gene.

One of ordinary skill in the art in the field of virology would know that the method suggested in *Clark* will not yield 100% recombinants, based, for example, on several earlier publications. See, for example, Clem, R.J., Fechheimer, M. and Miller, L.K. (1991) Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* v. 254, pp. 1388-1390; Hershberger, P.A., Dickson, J.A. and Friesen, P.D. (1992) Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: Cell line-specific effects on virus replication. *Journal of Virology* v. 66, pp. 5525-5533; Clem, R.J. and Miller, L.K. (1993) Apoptosis reduces both the in vitro replication and the *in vivo* infectivity of a baculovirus. *Journal of Virology* v. 67, pp. 3730-3738.

Clem and Miller (1993) demonstrated that viruses lacking p35 could still produce infectious virus in Sf21 cells (SF9 cells were derived from this original line). Although the

reduction in virus titres (a measure of infectivity) compared between the wild type virus and the p35 mutant was 50- to 100-fold, this still resulted in a yield of $>10^7$ plaque-forming units per millilitre of medium. Similar results were reported by Hershberger *et al.* (1992), although in this study Sf21 cells were infected with virus using low, medium, and high doses and the results showed that the difference in virus yield approached 200-fold when the higher doses were used. These studies show that deletion of the p35 gene from the baculovirus does not completely prevent virus replication. Therefore, a method using this gene as a selectable marker to make recombinant viruses will always promote the replication of the parental host virus to some degree.

A publication by Kitts *et al.*, also cited in the Office Action mailed August 26, 2004, teaches a method of producing a recombinant baculovirus vector in insect cells that uses linearized baculovirus DNA in combination with standard transfer vectors, which results in only 30-40% of recombinant viruses in the first round of plaque purification. Although linearization of the proposed host virus genome using *Bsu36I* helps to reduce parental virus, Kitts *et al.* showed that there was still a background level of parental virus that ranged between from 1% to 14%. The results by Kitts *et al.* support the assertion that the virus suggested in *Clark* will not result in the production of recombinant viruses that are completely free of parental stock. Even though parental virus is severely curtailed in its ability to replicate in insect cells, some virus will persist in the population and will require screening out at a later stage at more time and expense to the user of the method. The parent invention is more efficient and advantageously avoids this greater expense and loss of time.

11. Patel *et al.* (hereinafter *Patel*) fails to teach, suggest, or provide motivation to derive the claimed invention and fails to render it obvious. One of ordinary skill in the art would not be motivated by the teachings in *Patel* to derive the claimed invention. *Patel* uses yeast cells for a completely different reason as compared to the intermediate cell of the present application. *Patel* uses the yeast cells for recombination (that is, to modify the baculovirus), but not for maintenance of the baculovirus. In *Patel*, the insertion of foreign DNA is not carried out in the yeast cells; it is carried out in insect cells. The virus produced in the yeast in *Patel* is still infectious and is not defective. Yeast cells can be used to make a recombinant virus, but the *Patel* system does not involve the use of a defective baculovirus intermediate. The recombinant virus is made in the yeast cells, then recovered as DNA, and introduced into the insect cells.

One of ordinary skill in the art would not be motivated to combine *Clark* and *Patel* to derive applicants' claimed invention in the manner described in the August 26, 2004, Office Action. *Patel,* for example on page 97, right-hand column, paragraph 3, discourages one of ordinary skill in the art to combine its teachings with those of *Clark*. This passage states that the yeast system allows for the rapid generation of recombinant virus without any background parental virus. This is also discussed at the top of page 103, right-hand column. *Patel* teaches using the yeast cell for recombination and fails to teach, suggest, or provide motivation to use the insect cells for this purpose. The recombination is carried out in yeast because, as *Patel* states, insect cells were known to produce parental contamination. In contrast, in the claimed

invention recombination takes place in insect cells. Accordingly, Patel teaches away from applicants' claimed invention.

Moreover, *Patel* fails to overcome the time consuming difficulties associated with the use of the insect cells. Using the yeast system means that for every recombinant virus that is made, the experimenter still has to introduce a transfer vector with the foreign gene into the yeast cell, which harbours the fully infectious baculovirus genome. Recombination and selection of the yeast colonies ensures that the foreign gene is inserted into the virus genome. However, the baculovirus DNA must be recovered from each yeast strain containing the recombinant virus. Further, *Patel* recommends that the DNA is purified on a sucrose gradient to ensure infectivity (see page 99, left-hand column, second paragraph). This considerably adds to the complexity of the system in *Patel*. In contrast, in applicants' invention as claimed, a stock of defective baculovirus DNA, purified once from bacteria, is combined with various transfer vectors to make recombinant viruses in insect cells. This is a considerably simplified and more efficient system that is easier to use than the system in *Patel*, which makes it suitable for high throughput production of recombinant viruses, which would be difficult if not impossible with the *Patel* system. Consequently, one of ordinary skill in the art would not be motivated to combine the teachings of *Clark* and *Patel* because there is little advantage in combining the *Patel* system with the *Clark* system as such combination would result in a system where virus DNA would have to be purified from the yeast cells for each recombinant virus to be made.

One of ordinary skill in the art would not be motivated by the teaching of *Clark* and *Patel*, separately or in combination, to produce vectors that can replicate in both insect cells and yeast cells. In fact, one of ordinary skill in the art would be motivated to avoid combining the teachings of *Patel* to *Clark*. Although someone could use the *Patel* technique in insect cells using the benefit of hindsight, *Clark* and *Patel* alone or in combination fail to teach, suggest, or provide motivation to derive the claimed invention. Even if the technique of *Patel* were to be used in the insect cells taught in *Clark*, *Clark* uses SF9 cells that have inherent problems associated with them, as discussed above. Thus, combining the technique of *Patel* with the teachings of *Clark* would not result in the claimed invention. *Patel* only teaches using insect cells to produce infectious DNA particles by repeated plaque purification (see page 99, last paragraph), also demonstrating the difficulties of using SF9 cells. There is no background in *Patel* exactly because yeast and not insect SF9 cells are used. The whole aim of the *Patel* reference is to avoid using insect cells (see page 97, right-hand column, last paragraph).

Thus, one of ordinary skill in the art would not be motivated to combine the teachings of *Clark* and *Patel*. Many problems are associated with the teachings of *Clark*. *Patel* only suggests using yeast to produce a recombination event, which, according to *Patel*, avoids the problems associated with the previously known techniques using insect cells. This directs one of ordinary skill in the art away from using insect cells for recombination and away from the claimed invention.

12. The publication by Kitts *et al.* (hereinafter *Kitts*) fails to teach, suggest or provide motivation to derive applicants' invention, as claimed, and fails to render it obvious. One of ordinary skill in the art would not be motivated to derive the claimed invention from *Kitts*. One of ordinary skill in the art would also not be motivated to combine the teachings of *Kitts* and *Patel* as suggested in the Office Action mailed August 26, 2004.

Kitts teaches a method of producing a recombinant baculovirus vector in insect cells that uses linearized baculovirus DNA in combination with standard transfer vectors, which results in the production of only 30-40% of recombinant viruses in the first round of plaque purification. The *Kitts* method requires that virus DNA is digested with Bsu*36I* prior to use. Some DNA will remain undigested after treatment with the restriction enzyme because it is impossible to drive any enzyme-mediated reaction to completion, that is, to digest every circular DNA molecule. An enzyme is a biological catalyst. A catalyst drives a reaction in a certain direction, but only alters the equilibrium of two states. This is known to one of ordinary skill in the art. Therefore, when virus DNA is digested with a restriction enzyme, a small proportion will always remain intact. This circular DNA remains highly infectious and can give rise to parental virus DNA when attempting to make a recombinant baculovirus.

One of ordinary skill in the art would know that linearization of virus DNA does not result in the advantages of applicants' claimed method. For example, Table 1 in *Kitts* shows that using BacPAK6 after digestion results in 95% recombinants, with no apparent parental virus. However, on average 5% of the progeny virus had neither a parental, nor recombinant

phenotype. The parental phenotype should have produced blue plaques in the presence of X-gal. The recombinant plaque phenotype comprises the production of polyhedra after replacement of the lacZ gene in BacPAK6 by the polyhedrin gene. These 5% of plaques (ranging from 1-14% over nine separate experiments) did not produce polyhedra or the beta galactosidase required to turn plaques blue. Therefore, although not true parental phenotype, they constitute unwanted baculoviruses.

In the results reported for BacPAK5 in Table 1 of *Kitts*, 37% of the progeny plaques were parental. BacPAK5 is similar to BacPAK6, but has the polyhedrin gene in place of lacZ. Selection for recombinant viruses is achieved by loss of polyhedra production in virus-infected cells. Further 8% of plaques were neither recombinant nor parental in nature. Thus, linearization of parental virus DNA lacks the advantages of applicants' claimed method for producing recombinant viruses.

As noted above, *Patel* fails to teach the use of insect cells for recombination and provides motivation to avoid using insect cells for this purpose. Thus, one of ordinary skill in the art would not be motivated to combine the teachings of *Kitts* and *Patel*.

13. One of ordinary skill in the art would not be motivated to combine the cited publications as suggested, and would not be motivated to derive the claimed inventions based on the teachings of the publications cited in the Office Action mailed August 26, 2004.

14. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any patent issuing on this application.

RDPossee

Robert David Possee, Ph.D.

17/01/05

Date

CURRICULUM VITAE

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EDUCATION:

1968-1975	Secondary Education	9 O levels, 3 A levels, 1 S level
1975-1978	University of Birmingham	B.Sc. (Hons) 2.i, Biological Sciences
1978-1981	University of Warwick	Ph.D., Virology
	Supervisor Prof. N.J. Dimmock	
	PhD thesis:"The Mechanism of the Neutralization of Influenza Viruses by Antibody".	

PROFESSIONAL CAREER:

1981-present, NERC CEH Oxford (formerly Institute of Virology and Environmental Microbiology/Institute of Virology).

1981-1984, appointed Higher Scientific Officer (HSO) in Dr David Kelly's group as part of a project funded by NRDC to investigate the use of cell culture to produce baculoviruses for insect pest control. My specific duties were to develop molecular methods for the analysis of baculoviruses and novel insect cell lines. This laid the foundation for later work on virus expression vectors, recombinant protein production in cell culture and work on other baculoviruses such as *Panolis flammea* and *Mamestra brassicae* NPVs.

1984-1986, appointed Project Leader (HSO) of Baculovirus research group after departure of David Kelly. Initiated work on baculovirus expression vector system, which was disseminated within the Institute and remains a staple science at Oxford to the present day.

1986-1990, promoted to Senior Scientific Officer (SSO). Continued work on baculovirus expression vectors. Highlights include elucidation of the polyhedrin and p10 gene promoter structure and construction of expression vectors appropriate for the production of polyhedrin positive viruses and production of more than one foreign protein. This technology was key to the later development of recombinant baculovirus insecticides containing genes encoding insecticidal products.

1990-1999, promoted Unified Grade 7 (Principal Scientific Officer). A period that saw the construction and field testing of a recombinant baculovirus containing a gene encoding an insect-specific scorpion toxin gene. The completion of the first baculovirus genome sequence laid the foundation for future studies on genome function. An improved method for producing recombinant baculoviruses was developed and licensed to three companies. In 1992, I co-authored the first book detailing the necessary technologies required to undertake baculovirus expression vector work. After years of speculation, it was also discovered that insects can

harbour baculoviruses as persistent infections (at least in the laboratory, but see below for further work), challenging the dogma that virus occlusion bodies are the sole means of survival in the environment. The mechanism whereby baculoviruses liquefy their hosts was also revealed to involve at least two gene products: chitinase and cathepsin.
In 1993 I undertook a period of sabbatical leave in the laboratory of Professor Lois K. Miller in Athens, Georgia, USA. This provided a valuable insight to how one of the most successful scientists in the field operated and gave me the opportunity to develop some research ideas in the laboratory. I was also appointed visiting professor at Oxford Brookes University in 1998.

1999-present, Individual Merit Promotion (Band 3). This award has given me the freedom to pursue further novel lines of research, which in the face of a declining science budget, would have been difficult to attempt. Of particular current interest is the dissection of heterogeneous baculovirus populations by whole genome cloning in bacteria. This has revealed extensive virus population diversity in natural isolates, without the need for further amplification in insect hosts in the laboratory, which may introduce artifacts. A spin-off from this approach has been the development of an improved method for making recombinant baculovirus expression vectors, which was patented. I am currently developing DNA Microarray methods for analysing baculovirus gene expression. A collaborative project on baculovirus latency with Dr Rosie Hails and Prof. Linda King has demonstrated that wild populations of insects may also harbour persistent infections and offer an alternative way for the virus to survive in the field when host numbers are low.

2002-2003, appointed Deputy Director, CEH Oxford

2003-2004, Acting Director, CEH Oxford

2004 – present, Head of Department, CEH Oxford

SUPERVISION/MANAGEMENT OF STAFF:

Since 1984, I have supervised 4 science budget supported core research staff, 20 post-doctoral research fellows, 19 graduate students (PhD/D.Phil) and 4 research assistants. I have also hosted 7 visiting scientists from overseas. I have also had other management responsibilities involving the workshop staff at CEH Oxford and a photographer.

MANAGEMENT TRAINING:

Attended weeklong residential JTS course on Development for Senior Managers in 1998 and a half day course on communicating with the media. More recently, attended a one-day training course on safety for senior staff in Swindon.

TEACHING EXPERIENCE:

I have taught on undergraduate courses at Oxford University and Oxford Brookes University. I have also served as an external examiner for the University of London, University of Southampton, and as an internal examiner for the University of Oxford. PhD theses have also been examined from France and India.
Other extramural teaching has involved assisting the running of practical workshops on baculovirus expression vectors in Oxford, Paris, Argentina and Brazil

SCIENTIFIC MEETINGS ORGANISED:

I co-organised two International Workshops on Baculovirus Expression Vectors, in Oxford in 1988 and 1990. I also co-organised the Baculovirus workshop at the International Congress of Virology, Glasgow, 1993. I was a member of the Scientific Advisory Committee for Baculovirus and Insect Cell Gene Expression Conferences at Pinehurst, NC USA in 1995 and in Jersey in 1997. Large DNA

virus workshops have also been organized at various meetings of the Society for General Microbiology in the UK.

EDITORIAL BOARDS/INTERNATIONAL COMMITTEES:

I have been a member of the Virology Editorial Board since 1988; similarly for Journal of General Virology 1989 - 1992 and the Insect Virus Editor for the same journal since 1995. I was a member of the ICTV Baculovirus study group, 1990-1996 and am currently a scientific advisor to the EU Animal Cell Technology Industrial Platform (ACTIP).

CURRENT MEMBERSHIPS OF PROFESSIONAL SOCIETIES:

American Society for Virology
Society for General Microbiology
Society for Invertebrate Pathology
American Society for Microbiology
Biochemical Society
European Society for Animal Cell Technology UK

GRANTS/CONTRACTS AWARDED:

1. NERC Special Topic Award (1986-1989)
"Risk Assessment of the Release into the Environment of Genetically Engineered Baculovirus Insecticides". £60,000 (1 HSO/PDF)

2. NERC CASE Studentship (1986-1989)
CASE award with Professor R. Southwood, Dept of Zoology, Oxford University
"Molecular Biology of *Autographa californica* nuclear polyhedrosis virus"

3. Department of the Environment (1986-1989)
"The Risk Assessment of Genetically Engineered Baculovirus Insecticides"
£200,000 (1HSO/PDF, 1 SO/technician) + equipment (ABI DNA sequencer, £86,000).

4. NERC CASE Studentship (1987-1990)
CASE Award with Dr L.A. King, Oxford Brookes University
"Biological and Genetic Diversity of Baculoviruses"

5. Wellcome Environmental Health (1988-1991)
"The development of genetically engineered baculoviruses as novel, safe, specific
and environmentally acceptable insecticides" £200,000 (2 HSO/PDFs).

6. EC Biotechnology Action Programme (1989-1991)
"Risk Assessment of the Field Use of Genetically Engineered Baculoviruses"
£45,000 (1SSO/PDF). In collaboration with Dr J. Vlak, Wageningen, Netherlands.

7. NERC CASE Studentship (1989-1992)
CASE award with Dr L.A. King, Oxford Brookes University
"Latent baculovirus infections in field and laboratory insect populations"

8. NATO Collaborative research grant - for travel (1990-1994)
"Development of genetically engineered baculovirus insecticides containing modified JHE genes"£5,000.
In collaboration with Dr S. Maeda and Prof. B. Hammock, Davis, California.

9. Department of the Environment (1990-1993)
"Risk assessment of baculovirus insecticides" £379,000 (3 HSO/PDF).

10. NERC CASE Studentship (1991-1994)
NERC CASE award with Dr L.A. King Oxford Brookes University
"Analysis of baculovirus late gene expression factors"

11. Pfizer (1991-1994)
DPhil studentship
"Baculovirus expression vectors" £53,000.

12. Glaxo (1991-1994)
DPhil studentship
"Production of recombinant proteins using baculoviruses and insect cells" £50,000.
13. EC BRIDGE Programme (1992-1994)
"Biosafety of genetically modified baculoviruses" £85,000 (1HSO/PDF)
In collaboration with Dr J. Vlak and Dr J. Huber, Darmstadt, Germany.
14. NERC CASE Studentship (1992-1995)
CASE award with Dr L.A. King, Oxford Brookes University
"Baculovirus molecular biology"
Student resigned studentship in first year; award terminated
15. EC BIOTECH Programme (1993-1996)
"Baculovirus RNA polymerases" £135,000 (1 HSO)
In collaboration with: Dr D Knebel-Moersdorf, Cologne, Germany; Dr M. Lopez-Ferber, San Christol, France.
16. NERC CASE Studentship (1993-1996)
CASE award with Dr L.A. King, Oxford Brookes University
"The role of chitinase in the baculovirus infection in insects"
17. NERC Faraday Studentships (1993-1996)
2 awards with Dr L.A. King, Oxford Brookes University and Dr J. Windass (Zeneca Agrochemicals) "Genetic engineering of baculovirus insecticides"
2 awards with Professor D.H.L. Bishop, IVEM, Dr A. Kingsman, Oxford University and Dr T. French, British Biotechnology Ltd.
"Baculovirus expression vectors"
18. EC BIOTECH Programme (1994-1996)
"Minireplicon baculovirus expression vectors" £102,000 (1HSO)
In collaboration with Dr Just Vlak, Wageningen, Netherlands; Dr Dagmar Knebel-Moersdorf, Cologne, Germany; Dr Miguel Lopez-Ferber, San Christol, France; Dr Christian Oker-Blom, Turku, Finland.
19. Pfizer (1994 - 1997)
PhD studentship "Baculovirus expression vectors" £50,000 (PhD)
20. NERC CASE Studentship (1995-1998)
CASE award with Dr L.A. King, Oxford Brookes University
"Genetic and biological analysis of host range determinants in baculoviruses".
21. NERC Small Research Grant (1996-1997)
"Analysis of field collected insects for the presence of persistant baculovirus infections" £22,777.
With Dr L.A. King, Oxford Brookes University
22. NERC Standard Research Grant (1996-1998)
"Evolutionary and biological significance of baculovirus chitinases"
With Dr L.A. King, Oxford Brookes University
23. BBSRC Standard Research Grant (1996-1999)
"Baculovirus RNA polymerases" £138,000
With Dr L.A. King, Oxford Brookes University
24. NERC EDGE Programme Grant (1998-2001)
"Pathogen variability and dynamics in insect populations" £305,167
With Drs J.S. Cory and R.S. Hails (IVEM and Drs A.D. Watt and S. Hartley (ITE Banchory).
25. NERC EDGE Programme Grant (1998-2001)
"Genetic variation and the dynamics of pathogens in host-pathogen interactions" (£250,000 with Dr J.S. Cory (IVEM); Prof. M Begon and Dr D.J. Thompson (Liverpool).
26. EC BIOTECH FP IV (1998-2000)
"Baculovirus surface display - Developments and applications" £372,400 total (£108,500 [UK award] with Dr I.M. Jones (IVEM); Dr C. Oker-Blom (University of Helsinki); Dr R. Grabherr (Institute of Applied Microbiology, Vienna; Dr J. McCafferty (Cambridge Antibody Technology Ltd.
27. HSE (2001 - 2003)

"Development of an air sampling/biosensor system to detect genetically modified viruses" £35,000.

28. CEH Integrating fund (2001 - 2004)

"Interactions between viruses and lepidopteran larvae in different stress states" £150,000. Jointly held with Dr Daniel Osborn, CEH Monks Wood.

29. NERC CEH New blood position (2001 – 2004)

"Bioinformatics" £150,000. Jointly with Prof. Mark Bailey.

30. NERC Innovation fund (2000 – 2002)

"Development of baculovirus expression vectors" £52,337

31. NERC non-thematic (2001 - 2004)

"The ecological and evolutionary significance of latent baculovirus infections in insects". £282,846. Jointly held with Dr Rosie Hails (CEH), Prof. Linda King (Oxford Brookes University) and Dr Steve Sait (University of Liverpool).

32. OST Molecular Infrastructure Award (2001)

£750,000. Submitted on behalf of CEH and in collaboration with Prof. Mark Bailey (CEH Oxford).

PUBLICATIONS

1. **Possee, R. D. and Dimmock, N. J. (1981).** Neutralization of influenzavirus by antibody: attachment, uptake and uncoating of neutralized virus in chick embryo cells. In: *Genetic Variation Among Influenza Viruses*, pp. 473-480. ICN-UCLA Symposium XXI. Edited by D. P. Nayak & C. F. Fox. London: Academic Press.
2. **Possee, R.D. and Dimmock, N.J. (1981).** studies on the mechanism of neutralization of influenza virus by antibody: Evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus *in vivo* by inhibiting virion transcriptase activity. *Journal of General Virology* **58,** 373-386.
3. **Possee, R. D. (1986).** Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. *Virus Research* **5,** 43-47.
4. **Howard, S. C., Ayres, M. D. and Possee, R. D. (1986).** Mapping the 5' and 3' ends of *Autographa californica* polyhedrin mRNA. *Virus Research* **5,** 109-119.
5. **Matsuura, Y. Possee, R. D. and Bishop, D. H. L. (1986).** Expression of the S-coded genes of lymphocytic choriomeningitis arena virus using a baculovirus vector. *Journal of General Virology* **67,** 1515-1529.
6. **Moore, N. F., King, L. A. and Possee, R. D. (1986).** Viruses of Insects. *Insect Science Applications* **8,** 275-289.
7. **Matsuura, Y., Possee, R. D. Overton, H. A. and Bishop, D. H. L. (1987).** Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *Journal of General Virology* **68,** 1233-1250.
8. **Possee R. D. and Howard, S. C. (1987).** Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* **15,** 10233-10248.
9. **Possee R.D., Cameron, I.R., Allen, C.J. and Bishop, D.H.L. (1988).** Experiences with the first field release of genetically engineered viruses. In: *Viren und Plasmide in der Umwelt.* Schriftenreihe des Vereins fur Wasser-, Boden- und Lufthygiene **78,** p165-186. Eds. J.M. Lopez Pila, E. Seber and K. Jander. Gustav Fischer Verlag, Stuttgart/NewYork.
10. **Possee, R. D. and Kelly, D. C. (1988).** Physical maps and comparative DNA hybridization of *Mamestra brassicae* and *Panolis flammea* nuclear polyhedrosis virus genomes. *Journal of General Virology* **69,** 1285-1298.
11. **Bishop, D.H.L., Entwistle, P.F., Cameron, I.R., Allen, C.J. and Possee, R.D. (1988).** Field trials of genetically-engineered baculovirus insecticides. In: *The Release of Genetically-Engineered Micro-organisms* Eds. M. Sussman, C.H. Collins, F.A. Skinner and D.E. Stewart-Tull. Academic Press London.
12. **Weyer, U. and Possee, R.D. (1988).** Functional analysis of the p10 gene 5' leader sequence of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* **16,** 3635-3653.
13. **Bishop, D.H.L., Entwistle, P.F., Cameron, I.R., Allen, C.J. and Possee, R.D. (1988).** Genetically engineered baculovirus insecticides. *Aspects of Applied Biology* **17,** 385-395.
14. **Weyer, U. and Possee, R.D. (1989).** Analysis of the promoter of the *Autographa californica* nuclear polyhedrosis virus p10 gene. *Journal of General Virology* **70,** 203-208.
15. **Cameron, I.R. and Possee, R.D. (1989).** Conservation of polyhedrin gene promoter function between *Autographa californica* and *Mamestra brassicae* nuclear polyhedrosis viruses. *Virus Research* **12,** 183-199.
16. **Oakey, R., Cameron. I.R., Davis, B., Davis, E. and Possee, R.D. (1989).** Nucleotide sequence and transcription mapping of the polyhedrin gene of the *Panolis flammea* nuclear polyhedrosis virus . *Journal of General Virology* **70,** 769-775.
17. **Possee, R.D. and Bishop, D.H.L. (1989).** Areas of uncertainty in the uncontained use of modified organisms in the environment: The viruses from the molecular viewpoint. Proceedings of the European Biosafety Workshop: The Uncontained Use of Modified Organisms in the Environment.
18. **Cameron, I.R., Possee, R.D. and Bishop, D.H.L. (1989).** Insect cell culture technology in baculovirus expression systems. *Trends in Biotechnology* **7,** 66-70.
19. **Bishop, D.H.L. and Possee, R.D. (1990).** Baculovirus expression vectors. *Advances in Gene Technology* **1,** 55-90.
20. **Possee, R.D., Allen, C.J., Entwistle, P.F. Cameron, I.R. and Bishop, D.H.L. (1990).** Field trials of genetically engineered baculovirus insecticides. In: *Risk Assessment in Agricultural Biotechnology*, Proceedings of the International Conference, (J.J. Marois and G. Bruening, eds.) University of California.

61. **Possee, R.D., Cory, J.S., Hirst, M. and Bishop, D.H.L. (1993).** Field tests with genetically engineered baculoviruses. In: *BCPC monologue no. 55, Opportunities for molecular biology in crop protection*. (Eds. D.J. Beadle, D.H.L. Bishop, L.G. Copping, G.K. Dixon, D.W. Holloman).

62. **King, L.A., Possee, R.D., Atkinson, A., Palmer, C., Marlow, S., Pickering, J. and Beadle, D. (1994).** Advances in Insect Virology. In: *Advances in Insect Physiology* **25**, 1-73. Academic Press. London.

63. **Possee, R.D. and King, L.A. (1994).** Molecular approaches to the design of biotic crop protection agents. (1994). In: *Molecular Perspectives in Crop Protection* (Eds. G. Marshall and D. R. Walters) pp 68-97. Chapman and Hall.

64. **King, L.A., Mann, S.G., Lawrie, A.M. and Possee, R.D. (1994).** Production and isolation of recombinant baculoviruses. In: *Cell Biology Handbook* (Ed. K. Celis) Academic Press, New York. pp. 148-154.

65. **Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M. and Possee, R.D. (1994).** The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586-605.

66. **Hughes, D.S., Possee, R.D. and King, L.A. (1994).** Quantitation of latent *Mamestra brassicae* nuclear polyhedrosis virus DNA. *Journal of Virological Methods* **50**, 21-28.

67. **Bonning, B.C., Roelvinck, P.W., Vlak, J.M., Possee, R.D. and Hammock, B.D. (1994).** Superior expression of juvenile hormone esterase and beta-galactosidase from the basic protein gene promoter of *Autographa californica* nuclear polyhedrosis virus compared to the p10 and polyhedrin gene promoters. *Journal of General Virology* **75**, 1551-1556.

68. **Cory, J.S., Hirst, M.L., Williams, T., Hails ,R.S., Goulson, D., Green, B.M., Carty,T.M., Possee, R.D., Cayley, P.J. and Bishop, D.H.L. (1994).** Field trial of a genetically improved baculovirus insecticide. *Nature* **370**, 138-140.

69. **Possee, R.D. (1995).** Risk assessment and field trial with a genetically modified baculovirus insecticide. Proceedings from: *Pan-European conference on the potential long-term ecological impact of genetically modified organisms.* pp.147-163. Council of Europe Press.

70 **Higgs, S., Olsen, K.E., Klimowski, L., Powers, A.M., Carlson, J.O., Possee, R.D. and Beaty, B.J. (1995).** Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Sindbis virus vector. *Insect Molecular Biology* **4**, 97-103

71. **Hawtin, R.E., Arnold, K., Ayres, M.D., Zanotto, P.M de A., Howard, S.C., Gooday, G.A., Chappell, L.H., Kitts, P.A., King, L.A., and Possee, R.D. (1995).** Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212**, 673-685

72. **Bishop, D.H.L., Hirst, M.L., Possee, R.D. and Cory, J.S. (1995).** Genetic engineering of microbes: Virus insecticides - a case study. In Fifty Years of Microbials (Eds., Darby, G.K., Hunter, P.A. and Russell, A.D.) SGM Symposium Proceedings, Bath 1995, pp. 249-277.

73. **Merryweather-Clarke, A.T., Hirst, M.L. and Possee, R.D. (1995).** *In vivo* recombination between genetically modified and unmodified *Autographa californica* nuclear polyhedrosis virus in *Trichoplusia ni* larvae. *Acta Virologica* **38**, 311-315.

74. **Volkman, L.E., Blissard, G.W., Friesen, P., Keddie, B.A., Possee, R.D. and Thielmann, D.A. (1995).** Family Baculoviridae: In: *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*, pp. 104-113. Edited by F.A. Murphy, C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo and M.D. Summers. Springer-Verlag Wien, New York.

75. **Merrington, C.L., Kitts, P.A., King, L.A. and Possee, R.D. (1996).** An *Autographa californica* nucleopolyhedrovirus lef-2 mutant: consequences for DNA replication and very late gene expression. *Virology* **217**, 338-348.

76. **Possee, R.D. and Stacy, G. (1996)** Safety aspects of insect cell culture. In Insect cell cultures - fundamental and applied aspects. (Eds. J.M. Vlak, C.D. de Gooijer, H.G. Miltenburger and J. Tramper). Kluwer Academic Publishers.

77. **King, L.A. and Possee, R.D. (1997).** A future for baculovirus insecticides? Focus on Biopesticides January 1997.

78 **Hughes, D.S., Possee, R.D. and King, L.A. (1997).** Evidence for the presence of low-level,persistent baculovirus infection of *Mamestra brassicae* insects. *Journal of General Virology* **78**, 1801-1805.

40. **Kitts,.P.A., Ayres, D. and Possee, R.D. (1990).** Linearization of baculovirus DNA enhances the recovery of recombinant expression vectors. *Nucleic Acids Research,* **18**(19), 5667-5672.

41. **Weyer, U. and Possee, R.D. (1991).** A baculovirus dual expression vector derived from the *Autographa californica* nuclear polyhedrosis virus polyhedrin and p10 promoters: co-expression of two influenza genes in insect cells. *Journal of General Virology* **72**, 2967-2974.

42. **Possee, R.D., Bonning, B.C. and Merryweather, A.T. (1991).** Expression of proteins with insecticidal activities using baculovirus vectors. In: *Progress in recombinant DNA technologies and applications* (A. Prokop, ed.), *Annals of the New York Academy of Sciences* **646**, pp 234-239. USA.

43. **Bonning, B.C., Hammock, B.D., Hirst, M. and Possee, R.D. (1992).** Further development of a recombinant baculovirus insecticide expressing the enzyme juvenile hormone esterase from *Heliothis virescens. Insect Biochemistry and Molecular Biology* **22**, 453-458.

44. **Bonning, B.C., Merryweather, A.T. and Possee, R.D. (1991).** Genetically engineered viral insecticides. *Agricultural Biotechnology News and Information* **3**, 29-31.

45. **Stewart, L.M.D., Hirst, M., Lopez-Ferber, M., Merryweather, A.T., Cayley, P.J., and Possee, R.D. 1991.** Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature 352,* 85-88.

46. **Atkinson, A.E., Bermudez, I., Darlinson, M.G., Barnard, E.A., Earley, F.G.P., Possee, R.D., Beadle, D.J. and King, L.A. (1992).** Assembly of functional GABAA receptors in insect cells using baculovirus expression vectors. *Neuroreport* **3**, 597-600.

47. **Hawtin, R.E., King, L.A. and Possee, R.D. (1992).** Prospects for the development of a genetically engineered baculovirus insecticide. *Pesticide Science* **34**, 9-15.

48. **Roelvink, P.W., van Meer, M.M.M., de Kort, C.A.D., Possee, R.D., Hammock, B.D. & Vlak, J.M. (1992).** Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *Journal of General Virology,* **73**, 1481-1489.

49. **Possee, R.D., King, L.A., Weitzmann, M.D., Mann, S.G., Hughes, D.S., Cameron, I.R., Hirst, M.L. and Bishop, D.H.L. (1992).** Progress in the genetic modification and field-release of baculovirus insecticides. In: *The Release of Genetically Modified Microoganisms-REGEM 2*. pp 47-58. (D.E.S. Stewart-Tull and M. Sussman, eds.) Plenum Press, New York and London

50. **Bishop, D.H.L., Cory, J.S. & Possee, R.D. (1992).** The use of genetically engineered virus insecticides to control insect pests, In: *Release of genetically engineered and other microorganisms* pp 137-146. (M. Day and J.C Fry, eds.), Cambridge University Press.

51. **Weitzmann, M., Possee, R.D. and King, L.A. (1992).** Genetic analysis of two strains of *Panolis flammea* multiple nucleocapsid nuclear polyhedrosis virus. *Journal of General Virology,* **73**, 1881-1886.

52. **Possee, R.D. and Bishop, D.H.L. (1992).** Safety tests with genetically engineered baculovirus insecticides. In: Proceedings of the 2nd International Symposium on The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms, Goslar, Germany, 90-99.

53. **Bishop, D.H.L., Hill-Perkins, M.S., Jones, I.M., Kitts, P.A., Lopez-Ferber, M., Clarke (nee Merryweather), A.T., Possee, R.D., Pullen, J. and Weyer, U. (1992).** Construction of baculovirus expression vectors. In: *Baculovirus and Recombinant Protein Production,* (eds. Vlak, J.M., Schlaeger, E-J. and Bernard, A.R.), pp27-49, Roche, Basel, Switzerland.

54. **Hughes, D.S., Possee, R.D. and King, L.A. (1993).** Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* larvae. *Virology* **194,** 608- 615.

55. **Stewart, L.M.D. and Possee, R.D. (1993).** Baculovirus Expression Vectors. In: *Practical Approach Series* (A. Davison and R. Elliot, eds). pp. 227-256. Oxford University Press.

56. **Lopez-Ferber, M., Sisk, W.P. and Possee, R.D. (1995).** Baculovirus Transfer Vectors. In: *Baculovirus Expression Protocols. Methods in Molecular Biology* **39**, 25-63.

57 **Possee, R.D., Cayley, P.J., Cory, J.S. and Bishop, D.H.L. (1993).** Genetically engineered viral insecticides: New Insecticides with Improved Phenotypes. *Pesticide Science* **37**, 109-115.

58. **Hawtin, R.E. and Possee, R.D. (1993).** Genetic manipulation of the Baculovirus Genome for Insect Pest Control. In: *Parasites and Pathogens of Insects.* (N. Beckage, eds). Academic Press.

59. **Possee, R.D. (1993).** Viral approaches for insect control. In: *Advanced Engineered Pesticides* (L. Kim, ed).

60. **Kitts, P.A. and Possee, R.D. (1993).** A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* **14**, 810-817.

21. **Bishop, D.H.L. and Possee, R.D. (1989).** Planned release of an engineered baculovirus insecticide. UCLA Colloquium, *"New Directions in Biological Control"*. UCLA Symposium on Molecular and Cellular Biology. (P. Baker and P. Dunn, eds.), **112,** 100-110, Alan Liss Inc., New York.
22. **Bishop, D.H.L. and Possee, R.D. (1989).** Development of a novel genetically engineered viral insecticide. *Comptes Rendue de l'Academie d'Agriculture, France,* **75,** 141-152..
23. **Bailey, M.J. and Possee, R.D. (1989).** Manipulation of baculovirus vectors. In. *Gene expression in vivo. Methods in Molecular Biology* 17, Ed. E.J. Murray. Humana Press.
24. **Hammock, B., Szekacs, A., Hanzlik, Maeda, S., Philpott, M., Bonning, B. and Possee, R.D. (1990).** Use of transition state theory in the design of chemical and molecular agents for insect control. In: *Recent Advances in the Chemistry of Insect Control II,* (L. Crombie, ed.), pp 256-277. Royal Society of Chemistry Special Publication 79.
25. **Gearing, K. and Possee, R.D. (1990).** Functional analysis of a 603 nucleotide open reading frame in the EcoR I I fragment of *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* **71,** 251-262.
26. **Bishop, D.H.L., Harris, M.P.G., Hirst, M., Merryweather, A.T.and Possee, R.D. (1990).** The control of insect pests by viruses; opportunities for the future using genetically engineered virus insecticides. In: *Progress and prospects in insect control BCPC Monograph* **43,** 145-155.
27. **Possee, R.D., Harris, M.P.G., Hirst, M., Merryweather, A.T. and Bishop, D.H.L. (1990).** The deliberate release of genetically engineered virus insecticides into the environment. In. *COMETT Biotechnological Risk Management*: Protecting the European Environment.
28. **Possee, R.D., Merryweather, A.T., Weyer, U., Harris, M.P.G., Hirst, M. and Bishop, D.H.L. (1990).** The development and release of genetically engineered viral insecticides: A progress report 1986-1989. In: *Molecular Insect Science* (H.H. Hagedorn *et al.*, eds), Plenum Press, New York.
29. **Merryweather, A.T., Weyer, U., Harris, M.P.G, Hirst, Booth,T. and Possee, R.D. (1990).** Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. Kurstaki HD-73 delta endotoxin. *Journal of General Virology* **71,** 1535-1544.
30. **Hammock, B.D., Wroblewski, V., Harshman, L., Hanzlik, T.,Maeda, S., Philpott, M., Bonning, B.C. and Possee, R.D. (1990).** Cloning, expression and biological activity of the juvenile hormone esterase from Heliothis virescens. In: *Molecular Insect Science* (H.H. Hagedorn *et al.*, eds), Plenum Press, New York.
31. **Weyer, U., Knight, S. and Possee, R.D. (1990).** Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. *Journal of general Virology* **71,** 1525-1534.
32. **Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N. and Maeda, S. (1990).** Expression and effects of the juvenile hormone esterase in a baculovirus vector. *Nature* **344,** 458-461.
33. **Hill-Perkins, M.S. and Possee, R.D. (1990).** A baculovirus expression vector derived from the basic protein promoter of *Autographa californica* nuclear polyhdrosis virus. *Journal of general Virology* **71,** 971-976.
34. **Bishop, D.H.L., Hill-Perkins, M.S., Takehara, K., Urakawa, T., Weyer, U. and Possee, R.D. (1990).** The development and use of baculovirus vectors for multigene expression. In: *Recombinant Systems in Protein Expression* (K.K. Alito, M-L. Huhtala, Knowles, J. and A. Vaheri, eds.) pp56-61, Elsevier, Amsterdam.
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36. **Possee, R.D. and Bishop, D.H.L. (1992).** Baculovirus expression systems for insect cells. In: *Transgenesis-Applications of Gene Transfer* (J.A.H. Murray, ed.) pp 105-129 Open University Press.
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BOOK

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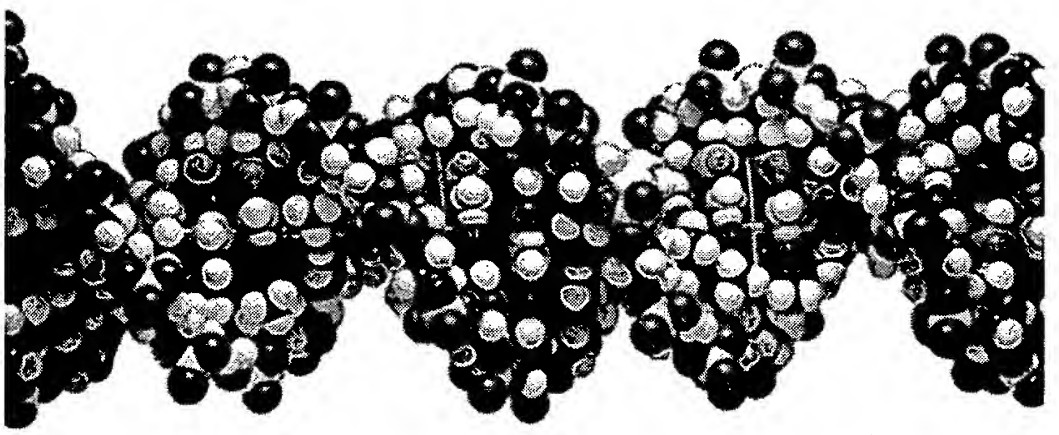
NCBE | Safety in the school laboratory

Working with DNA

2. Naked DNA



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DNA only gains a biological function by being inserted into a living cell. Hence work with DNA itself ('naked' DNA) is not generally thought to constitute a safety hazard even if new nucleic acid molecules are formed. Risks associated with most activities that might foreseeably be undertaken with naked DNA in school laboratories, such as gel electrophoresis, cutting with restriction enzymes, ligation and the polymerase chain reaction (PCR) can therefore be controlled by normal good laboratory practice.

The one exception to this is full length copies of viral DNA that are infectious in their own right. These are legally regarded as microorganisms even when they are not encapsulated or enveloped [18]. This means that if full-length viral DNA (such as DNA from phage lambda) were to be combined with DNA from other sources, a genetically-modified organism would have been created. For such work to be undertaken, the premises would have to be registered with the **Health and Safety Executive** (HSE). A brief summary of the regulations governing such work is given in section 3.3 below on 'Transformation'.

2.1 DNA extraction

Simple practical tasks such as the extraction of DNA from microorganisms, plant or animal tissue *e.g.*, fish sperm, may all be done following the relevant good laboratory health and safety precautions. For instance, where microorganisms are involved it is important to observe good microbiological practice.

If DNA is to be extracted from human tissue *e.g.*, cheek cells, for amplification by the PCR, the sampling procedure must be designed to minimise the risk of the transmission of infective agents between participants (for example, students should only work with their own DNA samples). It should also be borne in mind that crude extracts of DNA may still contain allergens or toxins present in the source material and must therefore be handled appropriately *e.g.*, if the seeds of a plant that contains a toxic alkaloid have been used.

The extraction of DNA from calf thymus tissue is sometimes referred to in school texts, although since the advent of BSE and vCJD this should no longer be done, as there is a risk (albeit small) of accidental exposure to the infectious agent while the extract is being prepared.

2.2 DNA from laboratory suppliers

DNA from a variety of organisms is available from molecular biology and school suppliers. Sources include bacteriophage lambda, salmon sperm and even cloned human DNA. While these can generally be regarded as safe, DNA from mammalian sources may not have been screened to ensure that it is free from contaminating viruses. It is therefore recommended that such material is not used in schools. In addition, full-length viral DNA that may have been genetically modified must not be used without prior registration with the HSE (see section 3.3, below).

2.3 Manipulation of DNA *in vitro*

With the exception of full-length viral DNA mentioned above, restriction and ligation of plasmid or other DNA, DNA gel electrophoresis and the polymerase chain reaction (PCR) may all be performed in a school laboratory.

2.4 Ethical considerations

Wider issues, including ethical concerns associated with the use of human DNA that may be construed as 'genetic tests' are beyond the scope of this safety document. Teachers should be aware that such issues may arise and ensure that any relevant practical procedure addresses these concerns by, for example, the random mixing of samples or the judicious selection of the DNA sequence to be investigated. Where appropriate, the relevant authorities may be consulted (*e.g.*, the **UK Human Genetics Commission**).

SECTION 3 ▶

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Naked DNA Effects

Last update: October, 2000

Genetic engineering sometimes involves "naked DNA," which is DNA stripped of the proteins that usually accompany it. Naked DNA can be created on purpose in the laboratory or can be unintentionally created during the genetic engineering process.

Using naked DNA can make it easier to transfer genes to a new cell during the genetic engineering process since the proteins that are usually attached to DNA can change the way it behaves in its new host - in some cases, making it much harder to successfully transfer a new trait into a plant genome.

But the same things that make naked DNA useful in the genetic engineering process also make it potentially dangerous. Freed of its regulating proteins, naked DNA can be taken up more easily by other cells. Once inside a cell, the DNA can integrate itself with that cell's own genes in new and unexpected ways, possibly producing biologically active levels of toxins or substances that can cause allergic reactions.

In the past, most scientists assumed that naked DNA was quickly broken down in digestive systems of animals when eaten, or in the environment by bacteria. But numerous recent experiments on genetically engineered vaccines have shown that naked DNA can be absorbed in ways and places previously believed not possible. For example, studies in the *International Journal of Pharmacology* and the *Journal of Clinical Investigation* have shown that naked DNA is readily absorbed when applied to human skin. And Terje Traavik, a professor at University of Tromsø in Norway, found in 1995 that naked viral DNA can be more infectious than the intact virus. Traavik tested "human polyoma virus" and found that when injected into rabbits, it had no effect. But when he injected bits of the naked viral DNA, it gave the rabbits a full-blown infection.

Many natural viruses and other pathogens affect only one or a few species. But genetic engineering breaks natural barriers that separate species. This means the genes transferred in the genetic engineering process, such as viral genes, can become "promiscuous," or able to incorporate themselves into many types of cells, triggering infections where they have never occurred previously.

Experts - Paul Billings, John Fagan, Michael Hansen

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Prevention of Apoptosis by a Baculovirus Gene During Infection of Insect Cells

ROLLIE J. CLEM, MARCUS FECHHEIMER, LOIS K. MILLER*

Programmed cell death is an active process of self destruction that is important in both the development and maintenance of multicellular animals. The molecular mechanisms controlling activation or suppression of programmed cell death are largely unknown. Apoptosis, a morphologically and biochemically defined type of programmed cell death commonly seen in vertebrates, was found to be initiated during baculovirus replication in insect cells. A specific viral gene product, p35, was identified as being responsible for blocking the apoptotic response. Identification of the function of this gene will allow further definition of the molecular pathways involved in the regulation of programmed cell death and may identify the role of apoptosis in invertebrate viral defense systems.

APOPTOSIS IS A MAJOR MECHANISM of physiologically relevant cell death in vertebrates. Cells undergoing apoptosis generate membrane-bound subcellular apoptotic bodies and activate an endogenous nuclease that cleaves the cellular chromatin DNA into discrete fragments. Apoptosis is ascribed roles in processes as diverse as embryogenesis, tumor regression, and cytolytic T cell–directed killing of virally infected cells (*1–5*). Programmed cell death also participates in insect development and tissue homeostasis (*6–9*), although chromatin digestion, the biochemical hallmark of apoptosis, has not been documented in invertebrate programmed cell death.

Our observation of apoptosis in baculovirus-infected insect cells arose during the characterization of a mutant of the baculovirus *Autographa californica* multiply-embedded nuclear polyhedrosis virus (AcMNPV). AcMNPV possesses a 128-kb circular DNA genome that replicates in the nucleus of its insect host cell (*10*). The infection is lytic; cell lysis normally occurs 72 or more hours after infection, after the virus particles are embedded in proteinaceous polyhedral occlusion bodies. During routine expression vector screening, the viral mutant, named the annihilator (vAcAnh), was isolated as a small plaque lacking occlusion bodies. In initial studies, vAcAnh was used to infect cell lines derived from three different species of lepidopteran insects. The mutant caused premature death of *Spodoptera frugiperda* (SF-21) (*11*) and *Bombyx mori* (BmN-4) (*12*) cells but not *Trichoplusia ni* (TN-368) (*13*) cells. We therefore propagated and titered vAcAnh using the TN-368 cell line.

The vAcAnh mutation was mapped by marker rescue to the AcMNPV Eco RI-S fragment (Fig. 1A), which contains the entire *p35* gene and a portion of the *p94* gene (*14*). The Eco RI-S fragment of vAcAnh was cloned and partially sequenced. A 754–base pair deletion was found that would result in the truncation of p35 by the removal of 132 amino acids from the carboxyl terminus and the addition of ten amino acids acquired by fusion into the adjacent hr5 region (Fig. 1, B and C).

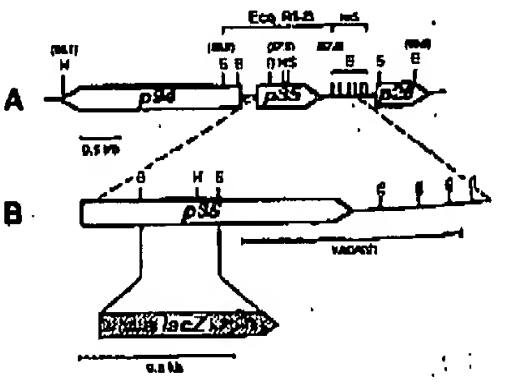
To confirm that the deletion of vAcAnh in *p35* was responsible for the annihilator phenotype, we inactivated *p35* of wild-type AcMNPV by inserting the *Escherichia coli lacZ* gene (Fig. 1B), resulting in the recombinant virus vP35Z. The phenotype of vP35Z is identical to that of vAcAnh (below), a result that indicates the presence of the intact *p35* gene is required to prevent premature death of AcMNPV-infected SF-21 cells.

SF-21 cells infected with wild-type AcMNPV and vAcAnh were examined by microscopy (Fig. 2). During a wild-type AcMNPV infection, small protrusions transiently appeared on the surface of the infected cells approximately 12 hours after infection (Fig. 2B). These protrusions normally disappeared, and by 24 hours occlusion bodies were visible in the nuclei. Cells infected with wild-type virus remained intact for 72 hours or more. This transient blebbing at 12 hours did not occur in TN-368 cells infected with either wild-type or vAcAnh (*15*). In vAcAnh-infected SF-21 cells, protrusions appeared on the surface of the cells (Fig. 2C) that were similar to protrusions observed in a wild-type infection. However, instead of disappearing with time, the blebbing process intensified and large, membrane-bound bodies arose and separated from the cells (Fig. 2D). The nucleus and cytoplasm of an affected cell were sectioned into these bodies until only a cluster of bodies remained. For an individual cell, the entire process of disintegration required approximately 1 to 2 hours. The subcellular bodies, which are morphologically similar to apoptotic bodies, remained over the next few days.

The mitochondria of blebbing cells remained functionally intact during the blebbing process. Staining with rhodamine 123, which accumulates in mitochondria with an active membrane potential (*16*), revealed energized mitochondria in the blebbing cells and in some of the bodies themselves (Fig. 2, E and F). Our results are similar to those of previous studies, which showed morphologically intact mitochondria in apoptotic cells and apoptotic bodies (*2–4*). These results suggest that the cells are undergoing an active process of cell death.

The nuclei of vAcAnh-infected SF-21 cells broke up into small fragments during the blebbing process (Fig. 2, G and H), and nuclear fragments were present in some of the bodies released by the dying cell. This type of nuclear disintegration is also observed in the process of apoptosis (*2–4*).

Because the morphological evidence (Fig. 2) indicated that vAcAnh-infected SF-21



C
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AAC CAA GTG CTA TAT GAC TCA TTT GTT TTT CAA AAC CGA AGT TGA
Asn Gln Val Leu Tyr Asp Ser Phe Val Phe Gln Asn Arg Thr *
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Fig. 1. Mapping and sequencing of the vAcAnh mutation and construction of vP35Z, the *p35-lacZ* insertion mutant (*24*). (A) Partial genome map of AcMNPV. The three major open reading frames in the region, which encode p94, p35, and p26 are indicated by arrows. Numbers in parentheses represent map units on the AcMNPV genome. The hr6 region is one of six homologous regions in the AcMNPV genome that are highly repetitive and contain multiple Eco RI sites (*25*). The restriction sites are as follows: B, Bcl I; E, Eco RI; H, Hind III; and S, Sal I. (B) Expanded view of the region surrounding the *p35* gene. The site of the *lacZ* insertion (hatched box) is indicated, as is the extent of the deletion in vAcAnh (line beneath the genome). (A) and (B) are drawn to scale, except for the *lacZ* insert, which is 4.2 kb in length. (C) Nucleotide sequence and predicted amino acid sequence of the region immediately surrounding the vAcAnh deletion. The arrow indicates the junction created between position +502 of *p35* (*14*) and position −296 of *p26* (*25*) (from the initiation codons). The asterisk indicates a stop codon.

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cells were undergoing an apoptosis-like response, we examined the chromatin DNA of wild-type and *p35* mutant-infected cells by agarose gel electrophoresis. A characteristic biochemical feature of apoptosis is the endonucleolytic cleavage of the cellular DNA into a chromatin ladder consisting of discrete fragments of oligonucleosomal lengths (*17*). The DNA of SF-21 cells infected with either of the two *p35* mutants, vAcAnh or vP35Z, was digested into fragments characteristic of a chromatin ladder (Fig. 3). This degradation began between 6 and 12 hours after infection and increased with time. As expected (*18*), the DNA of wild-type–infected SF-21 cells remained in a high molecular weight form over the course of the infection (Fig. 3), as did the DNA of TN-368 cells infected with the wild-type or either of the two mutants (*15*). The DNA of cells killed by freeze-thawing also remained in a high molecular weight form (Fig. 3), further indicating that *p35* mutant–induced DNA fragmentation was not simply due to rapid necrosis. In addition, cell viability, as determined by trypan blue exclusion, correlated with DNA fragmentation (legend to Fig. 3). The chromatin degradation and microscopy data (Fig. 2) indicate that premature death in *p35* mutant–infected SF-21 cells occurs by the process of apoptosis.

Thus, p35 blocks the apoptotic response that SF-21 cells mount in response to AcMNPV infection. Preliminary data indicate that p35 affects the rate at which host protein synthesis is curtailed and late viral gene expression is initiated (*15*). The transition from the early to the late phase of viral replication occurs approximately 6 hours after infection, the time at which apoptosis is initiated. Because RNA and protein synthesis are required for apoptosis to occur (*3, 4*), p35 may directly or indirectly interfere with the synthesis of cellular proteins that induce apoptosis. Transcription of *p35* occurs almost immediately on viral entry and continues throughout the course of the infection (*14, 19*). Thus, p35 is present by 6 hours after infection and may be required to maintain the block throughout the remainder of the infection process.

As determined by computer-assisted sequence analysis, the predicted p35 gene product (*14*) shares no discernible sequence relationship to any proteins in current gene databases, including the three other gene products that have been found to regulate apoptosis. Two of the genes known to regulate apoptosis, the proto-oncogene *bcl-2* and the Epstein-Barr virus latent gene *LMP 1*, block the apoptotic response of B lymphocytes by an unknown but apparently common mechanism (*20, 21*). The third gene, the tumor-suppressor gene encoding p53, induces apoptosis in a myeloid leukemic cell line, again by an unknown mechanism (*22*). All three of these genes regulate apoptosis in cells of the mammalian immune system. The *p35* gene, however, regulates apoptosis outside the immune system. Because the regulation of apoptosis is expected to be a critical factor in cell proliferation, and therefore in tumorigenesis, it will be important to identify the mechanism by which this protein controls cell death.

For the host organism, a cellular apoptotic response during viral infection probably has significant effects on viral pathogenesis. Apoptosis may have evolved as a primitive viral defense response in animals lacking humoral immune systems (*5, 23*). The amount of budded and occluded virus obtained from vAcAnh-infected SF-21 cells and of occluded virus obtained from *Spodoptera frugiperda* larvae (*15*) are dramatically reduced compared to amounts of viruses obtained in the wild-type strain. Thus, the presence of *p35* is expected to significantly increase the virulence of AcMNPV in at least some hosts. Overcoming this type of host defense response could contribute to the ability of AcMNPV to infect a broader range of insect cells.

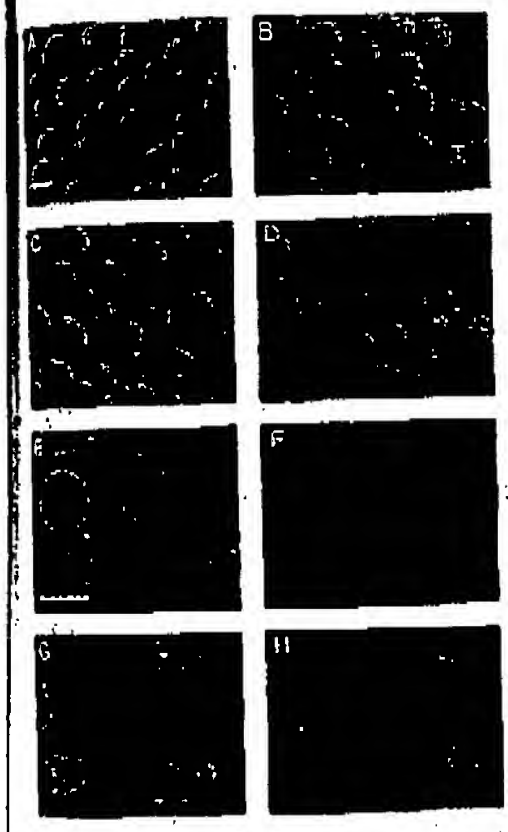

Fig. 2. Phenotype of vAcAnh at the microscopic level. (A to D) SF-21 cells were either mock-infected (A), wild-type AcMNPV-infected (B), or vAcAnh-infected (C and D) and observed at 12 hours after infection with differential interference contrast optics (*26*). Cells in early (C) and later (D) stages of blebbing are shown. Because the apoptotic response is not perfectly synchronous, some cells do not appear to be blebbing; even though <20% and <5% of the cells remain viable by 24 and 36 hours after infection, respectively (legend to Fig. 3). (E and F) Blebbing cells contain intact mitochondria, as judged by their ability to be stained with rhodamine 123. SF-21 cells infected with vAcAnh (12 hours after infection) were stained with rhodamine 123 and examined by phase-contrast (E) and flourescence (F) microscopy (*26*). The stain was confirmed to be specific for intact mitochondria by treating stained cells with the proton ionophore 2,4-dinitrophenol, which resulted in rapid destaining (*15*). (G and H) Nuclear fragmentation in blebbing cells. vAcAnh-infected SF-21 cells (12 hours after infection) were fixed, stained with the nuclear stain Hoechst G33342, and examined by phase-contrast (G) and fluorescence (H) microscopy (*26*). Bars, 20 μm.

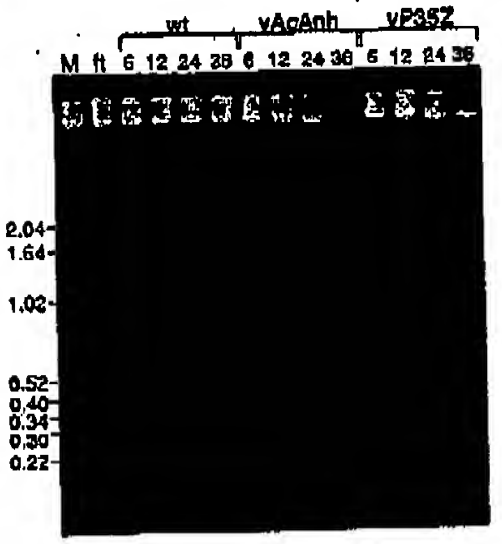

Fig. 3. Agarose gel showing DNA fragmentation in SF-21 cells infected with *p35* mutant viruses. Total DNA from mock-infected (M), freeze-thawed (ft), wild-type AcMNPV-infected, vAcAnh-infected, or vP35Z-infected cells was isolated at various times after infection, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining (*27*). Size markers are indicated to the left (in kilobases). Numbers at the top indicate hours after infection. The percentage of viable cells in each sample and the percentage of total DNA in fragments (in parentheses) was as follows (*27*): mock, 98.3% (0.0%); freeze-thawed, 1.6% (9.7%); wild type at 6 hours, 99.0% (0.0%); wild type at 12 hours, 99.2% (5.1%); wild type at 24 hours, 98.8% (7.1%); wild type at 36 hours, 97.5% (7.0%); vAcAnh at 6 hours, 98.8% (0.0%); vAcAnh at 12 hours, 62.5% (28.1%); vAcAnh at 24 hours, 17.2% (63.7%); vAcAnh at 36 hours, 4.7% (67.9%); vP35Z at 6 hours, 99.3% (0.0%); vP35Z at 12 hours, 67.6% (−7.0%); vP35Z at 24 hours, 20.2% (53.9%); and vP35Z at 36 hours, 6.5% (62.1%).

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24. The vAcAnh mutation was mapped to the Eco RI-S fragment (86.7 to 87.9 map units) by cotransfection of SF-21 cells with vAcAnh DNA and a series of recombinant λ DNAs containing the entire wild-type (L-1 strain) [H. H. Lee and L. K. Miller, *J. Virol.* 27, 754 (1978)] AcMNPV genome in overlapping segments. The vAcAnh *Eco* RI-S fragment was cloned into pBluescript (Stratagene, La Jolla, CA) and sequenced by the dideoxy chain termination method with Sequenase (U.S. Biochemical Corp., Cleveland, OH). vP35Z was constructed by replacing the Bcl I–Sal I fragment of *p35* with a Bam HI–Sal I fragment from plasmid pMC874 [M. J. Casadaban, J. Chou, S. N. Cohen, *J. Bacteriol.* 143, 971 (1980)], which contained the *lacZ* gene. The resulting recombinant plasmid, which had *lacZ* fused in-frame to the NH_2-terminus of *p35*, was used to construct the recombinant virus vP35Z by cotransfection of TN-368 cells with wild-type DNA. vP35Z was identified as a blue plaque in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and its DNA was characterized by restriction endonuclease analysis and Southern (DNA) blotting.
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27. SF-21 cells were infected as above (*26*) but with 60-mm tissue culture plates. After the indicated number of hours, the cells (4×10^6 per sample) were lysed in 0.4 M tris (pH 7.5), 0.5 M EDTA, 0.1% SDS, and 200 μg ml^{-1} proteinase K for 6 hours at room temperature. The lysates were phenol extracted and ethanol precipitated. Half of each sample was loaded on a 1.2% agarose gel and separated by electrophoresis overnight at 60 volts in buffer containing ethidium bromide. The gel was then treated for 6 hours with 20 μg ml^{-1} ribonuclease A in running buffer with ethidium bromide at room temperature. Freeze-thawed cells were subjected to three 5-min cycles in liquid nitrogen and a 37°C water bath before DNA isolation. To determine cell viability, we stained cells with 0.04% trypan blue in culture medium. Intact cells that excluded the dye were counted as viable, and the percentage of viable cells that remained was determined based on the original number of cells (approximately 1000) per unit volume. To determine the percentage of DNA in fragments, we scanned a Polaroid negative of the gel with Molecular Dynamics Computing Densitometer Model 300A and ImageQuant version 3.0. The lane containing the freeze-thawed cells was normalized to the mock lane, and lanes containing virus-infected cells were normalized to the 6-hour lane. The amount of DNA fragmentation in the vP35Z 12-hour sample in this particular experiment was atypical, as it was normally similar to vAcAnh at 12 hours.
28. We thank R. Rusmak for the gift and the naming of the annihilator mutant, B. G. Ooi for preliminary characterization of the mutant, S. Maeda and T. Morris for the BmN-4 cell line, D. O'Reilly for many insightful comments and suggestions, and S. Hilliard and J. Todd for excellent technical assistance. Supported in part by Public Health Service Grant AI23719 from the National Institute of Allergy and Infectious Diseases to L.K.M.

7 June 1991; accepted 13 September 1991

JOURNAL OF VIROLOGY, July 1993, p. 3730–3738
0022-538X/93/073730-09$02.00/0

Vol. 67, No. 7

Apoptosis Reduces both the In Vitro Replication and the In Vivo Infectivity of a Baculovirus

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Received 29 January 1993/Accepted 26 March 1993

Apoptotic programmed cell death occurs when the insect cell line SF-21, derived from *Spodoptera frugiperda*, is infected with mutants of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) which lack a functional *p35* gene. However, infection of the *Trichoplusia ni* TN-368 cell line with *p35* mutants does not result in apoptosis (R. Clem, M. Fechheimer, and L. Miller, Science 254:1388–1390, 1991). We have examined the effect of apoptosis on AcMNPV infections in cell lines and larvae of these two insect species. Production of viral progeny was significantly lower in SF-21 cells infected with *p35* mutants than in cells infected with wild-type (wt) or revertant viruses. Viral gene expression was abnormal in SF-21 cells infected with *p35* mutants; there was a delay in the transcription and translation of early and late viral genes, a lack of expression of very late genes, and a total cessation of protein synthesis late in the apoptotic process. In vivo analysis revealed that the dose of budded virus required for 50% lethality in *S. frugiperda* larvae was approximately 1,000-fold higher for *p35* mutants than for wt or revertant viruses. In contrast, the replication and infectivity of *p35* mutant viruses was equivalent to that of wt AcMNPV during infection of both TN-368 cells and *T. ni* larvae. Thus, the data indicate that a host apoptotic response provides protection against viral infection at the organismal level and that the *p35* gene constitutes a host range determinant for AcMNPV infection.

Apoptosis, a distinctive type of programmed cell death, was first described in 1972 by Kerr et al. (22) and is now widely recognized as being vital to a diversity of biological processes, including embryonic development, tissue homeostasis, tumorigenesis, and the lysis of virally infected cells by cytotoxic T lymphocytes (42). In addition to being important in organismal and cellular functions, apoptosis also appears to be an important factor in the replication strategies of a wide variety of eukaryotic viruses. A number of disparate viruses are known to trigger apoptosis during infection (14, 20, 24a, 28, 40), and members of at least three different virus families, *Herpesviridae*, *Adenoviridae*, and *Baculoviridae*, possess genes which can prevent apoptotic cell death of their host cells (2, 3, 8, 15, 43). Inhibition of apoptosis has also been correlated with viral latency (13a) and persistence (24a). The ability to block premature death of the host cell should provide significant advantages for most obligate intracellular parasites, although the extent of these advantages for lytic viruses has not been fully explored.

Apoptosis occurs upon infection of either the *Spodoptera frugiperda* SF-21 (3, 16) or the *Bombyx mori* BmN-4 (3) cell line with *Autographa californica* nuclear polyhedrosis virus (AcMNPV) mutants lacking a functional *p35* gene. In the case of SF-21 cells, the majority of the cells undergo apoptosis within 12 to 24 h postinfection (p.i.), and no occlusion bodies are formed (3). In contrast, infection of *Trichoplusia ni* TN-368 cells with *p35* mutants results in normal virus replication, including occlusion body production, during the very late phase of infection between 24 to 70 h p.i. (3, 16). Both the SF-21 and TN-368 cell lines are fully permissive for wild-type (wt) AcMNPV replication, whereas the BmN-4 cell line does not normally support productive AcMNPV replication, although significant expression from all three temporal classes of viral promoters is observed (30). In addition, the *p35*-homologous gene from the baculovirus *B. mori* nuclear polyhedrosis virus (BmNPV) also appears to be involved in preventing apoptosis in BmN cells, as infection with BmNPV mutants lacking the BmNPV *p35*-homologous gene results in a mixed phenotype, with some cells becoming apoptotic and others supporting full virus replication (21). However, although differences in the response of various cell lines to *p35* mutant infection have been observed (3, 16, 21), it is not known whether these differences are species specific or cell line specific.

The mechanism by which P35 acts in blocking apoptosis is not clear, although insights into its function are beginning to emerge. Infection of SF-21 cells with wt AcMNPV but not *p35* mutant viruses is able to block apoptosis triggered by a nonviral signal, actinomycin D, suggesting that P35 (possibly in conjunction with other viral gene products) is able to interact directly in the host apoptotic pathway rather than acting by preventing viral triggering of apoptosis (8). However, if actinomycin D is added to the cells prior to 5 h p.i., apoptosis results (8), indicating that viral gene expression is required for inhibition of cell death. Consistent with a role early in the infection process, the *p35* gene is transcribed at both early and late times in infection (10, 11, 19, 31). The predicted sequence of the P35 polypeptide (11) currently provides little or no help in understanding its function, although the C terminus of P35, including the last 12 amino acids, is known to be essential for function (16).

P35 is not the only baculovirus gene product able to block AcMNPV- or actinomycin D-triggered apoptosis; a second gene encoding a gene product with a zinc finger-like motif was identified in the genome of *Cydia pomonella* granulosis virus (CpGV) as a gene capable of blocking apoptosis triggered by infection with *p35* mutants of AcMNPV (8). Effectively this gene, known as *iap* (for inhibitor of apoptosis), can replace *p35* function in blocking apoptosis in SF-21 cells. A homolog of *iap* is also found in the AcMNPV

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genome, but the AcMNPV *iap*-homologous gene (Ac-*iap*) is unable to block apoptosis in the absence of *p35* (8). It may be that both Ac-IAP and P35 are required and interact to prevent apoptosis during AcMNPV infection of SF-21 cells; alternatively, Ac-IAP may have evolved a different function.

The role of apoptosis in the control of virus infection by cytotoxic T lymphocytes in vertebrates has led to the suggestion that apoptosis may have originally evolved as a defense response to viral infection in primitive animals lacking a humoral immune system (6, 27). The observation that apoptosis occurs in insect cells infected with *p35* mutants of AcMNPV provides an ideal model with which to test this hypothesis and also to explore the impact that apoptosis may have on viral infection in the absence of an antibody response. Very little is known concerning insect defenses against virus infection; although insect hemocytes can provide cell-mediated immunity to bacterial pathogens through phagocytosis or encapsulation (13), neither cell-mediated nor humoral immunity has been demonstrated against baculovirus infection in insects.

In a recent report, Hershberger et al. showed that disruption of *p35* results in reduced yields of progeny budded virus (BV) and decreased synthesis of late viral proteins in SF-21 but not in TN-368 cells (16). Here we confirm and extend the in vitro results of Hershberger et al. and further analyze the replication and infectivity of AcMNPV *p35* mutants in insect larvae. The infectivities of wt AcMNPV and the *p35* mutants were similar in *T. ni* larvae, and replication of these viruses, including the expression of selected viral transcripts and viral proteins, was normal in TN-368 cells. In *S. frugiperda* larvae and SF-21 cells, however, apoptosis had an adverse effect: *p35* mutants were significantly less infectious in vivo and were impaired in their replication, transcription, and protein synthesis in SF-21 cells. This finding represents the first experimental evidence at the organismal level that apoptosis provides host protection against viral infection and supports the hypothesis that apoptosis may have evolved as an antiviral response in primitive animals. The ability to block such a response also appears to be an important host range determinant in baculovirus infections. Finally, a delay in viral gene expression was observed in *p35* mutant-infected SF-21 cells, suggesting a role for P35 in the timely expression of early viral genes involved in blocking the apoptotic response of the cell.

MATERIALS AND METHODS

Viruses, cell lines, and insects. Wild-type (L-1 strain) AcMNPV (24) and the *p35*-revertant viruses vAnhHK5 and vP35ZRS were propagated in IPLB-SF-21 (SF-21) cells (41) by previously described methods (35). The AcMNPV *p35* mutants, annihilator (vAcAnh) and vP35Z (3), were propagated in TN-368 cells (17). BV titers for all of the viruses were determined by plaque assay using TN-368 cells as described previously (35). SF-21 and TN-368 cells were maintained at 27°C in TC-100 medium (GIBCO BRL Laboratories, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Intergen, Purchase, N.Y.) and 0.26% tryptose broth. *S. frugiperda* and *T. ni* eggs were kindly provided by W. Deryck Perkins (Agricultural Research Service, U.S. Department of Agriculture, Tifton, Ga.) and Beth Gray (Abbott Laboratories, Chicago, Ill.), respectively, and the insects were reared in individual cups on artificial diet (35) at 27°C under a 14-h/10-h light/dark cycle.

Construction of revertant viruses. vAnhHK5 was constructed by calcium phosphate cotransfection (35) of SF-21 cells with vAcAnh DNA and the lambda clone HK5 (37), which contains wt AcMNPV sequences from approximately 80 to 91 map units (including *p35*), and screening for viruses with an occlusion-positive plaque phenotype. vP35ZRS was constructed similarly except that vP35Z DNA and plasmid pRS, containing the *Eco*RI S fragment of wt AcMNPV from 86.8 to 87.9 map units (including *p35*) were used. The construction of both revertant viruses was verified by restriction enzyme analysis and Southern blotting.

Protein pulse labeling. SF-21 or TN-368 cells (10^6 of each) were mock infected with TC-100 or infected with wt AcMNPV, vAcAnh, or vP35Z at a multiplicity of infection (MOI) of 20 PFU per cell. After a 1-h adsorption period, the viral inoculum was removed and replaced with complete TC-100; the time of refeeding was taken as time zero. Two hours before the appropriate time point, the medium was removed and replaced with incomplete TC-100 lacking methionine. Because of the propensity for *p35* mutant-infected SF-21 cells and apoptotic bodies to lift off the plate, all of the SF-21 samples were refed by scraping the cells, transferring the cells and the culture supernatant to centrifuge tubes, centrifuging the cells 5 min at 1,000 × *g*, and gently resuspending the pellets in methionine-free TC-100. Following incubation for 1 h, 25 μCi of [^{35}S]methionine (New England Nuclear, Boston, Mass.) was added per plate or tube, and the cells were incubated for an additional hour. The monolayers or cell pellets were then washed and lysed in 50 μl of lysis buffer as described previously (35). The lysates were stored at −80°C until they were analyzed. Five microliters of each lysate was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (23) on 10% polyacrylamide gels and subjected to fluorography as described previously (35).

Primer extensions. SF-21 or TN-368 cells (6×10^6 of each) were mock infected or infected with wt AcMNPV or vP35Z at an MOI of 20 PFU per cell as described above. At 6, 12, 24, and 48 h p.i., the cells were harvested and total RNA was isolated by the guanidinium isothiocyanate method (1). The cells were harvested by scraping, and then both the cells and the culture supernatant were centrifuged as described above to avoid losing any apoptotic bodies. Twenty micrograms of RNA was hybridized to oligonucleotides complementary to the *egt*, capsid, or polyhedrin open reading frame (ORF) (see below) which had been 5′ end labeled with T4 polynucleotide kinase (GIBCO BRL). The oligonucleotide primers were then extended by using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), and the extended products were analyzed by urea-polyacrylamide gel electrophoresis and autoradiography.

The oligonucleotides used for primer extension were as follows: *egt*, 5′-AGTGCAAGCCAGCAGAG-3′, complementary to the sequence from +26 to +10 of the *egt* ORF (nucleotides 174 to 158 of reference 34); capsid, 5′-CGCC ATACCCACGGG-3′, complementary to the sequence from +27 to +13 of the *vp39* ORF (nucleotides 491 to 477 of reference 39); and polyhedrin, 5′-GGTACGCCCGATGGT GGG-3′, complementary to the sequence from +39 to +22 of the *polh* ORF (18).

Virus growth curves. SF-21 or TN-368 cells (2×10^6 of each) were infected with virus at an MOI of 20 PFU per cell as described above except that after removal of the virus inoculum, the cell monolayers were washed two times with TC-100 and then refed with 5 ml of TC-100. Immediately and at 12, 24, and 48 h p.i., 0.5 ml of the culture medium was removed and stored at 4°C until determination of titers. Samples were titered by plaque assay using TN-368 cells.

Larval bioassays. Various doses of BV (as determined by plaque assay using TN-368 cells) were injected into the hemocoel of developmentally staged larvae (25 per dose) within 24 h of molting into the penultimate larval instar (fifth-instar *S. frugiperda* or fourth-instar *T. ni*). Complete TC-100 was used to dilute the BV samples. Mock-infected insects were injected with the same volume of complete TC-100. Injected larvae were observed daily for mortality, as determined by overall appearance and lack of response to agitation, until either death or pupation occurred. Larvae which died prematurely due to injection mortality were not considered in the final results.

Occluded virus yields. Larvae which were injected with the highest dose of each virus and which died from viral infection were collected at the time of death and stored at −20°C until the end of the experiment. Each group of larvae was pooled and homogenized in 1% SDS (1 ml per larva), and occlusion bodies were counted in a hemocytometer following dilution in distilled water. Two independent counts were made for each sample. The number of occlusion bodies per insect was taken as being equivalent to the number of occlusion bodies per milliliter.

RESULTS

Protein synthesis in cells infected with *p35* mutant viruses. The *p35* mutant viruses which were used in these experiments were described previously (3). Briefly, vAcAnh was isolated as a spontaneous mutant and was found to have a deletion affecting the *p35* gene and the adjacent *hr5* region, while vP35Z was constructed by replacement of a portion of *p35* with the *Escherichia coli lacZ* gene.

To characterize the effect of apoptosis on Ac*M*NPV infection at the cellular level, we examined protein synthesis profiles of these two different *p35* mutants in SF-21 cells (which undergo apoptosis) and in TN-368 cells (which do not undergo apoptosis). The pattern of protein synthesis in SF-21 cells infected with either vAcAnh or vP35Z differed substantially from that of cells infected with wt Ac*M*NPV (Fig. 1A). Although there was very little evidence of virally induced protein synthesis in cells infected with vAcAnh or vP35Z, two new proteins of M_r approximately 33,000 and 37,000 (33K and 37K proteins) were induced, but their synthesis appeared to be delayed relative to that of wt-infected cells. Both of these proteins are early proteins; their synthesis is observed in cells infected with wt Ac*M*NPV in the presence of aphidicolin (38), which blocks late gene expression by blocking DNA replication, and in cells infected with *ts8*, a conditionally lethal mutant blocked in DNA synthesis as a result of a mutation in a helicase-homologous gene (12, 25). In addition, the 33K polypeptide is clearly observed in SF-21 cells infected with the mutant *ts*B821, a temperature-sensitive mutant which is blocked in the early phase (29). There was no evidence of late (e.g., gp67 [44]) or very late (e.g., polyhedrin, 30K) protein synthesis in *p35* mutant-infected SF-21 cells (Fig. 1A). Host protein synthesis was not specifically shut off in *p35* mutant-infected SF-21 cells, as is normally the case for Ac*M*NPV infection. However, there was a general decline in protein synthesis between 18 and 24 h p.i., and by 48 h p.i., no protein synthesis was detected (Fig. 1A). In wt-infected cells, host protein synthesis began to decline approximately 18 to 24 h p.i., and virus-specific protein synthesis increased until, by 48 h p.i., polyhedrin synthesis predominated (Fig. 1A).

In contrast, the protein synthesis profiles of TN-368 cells

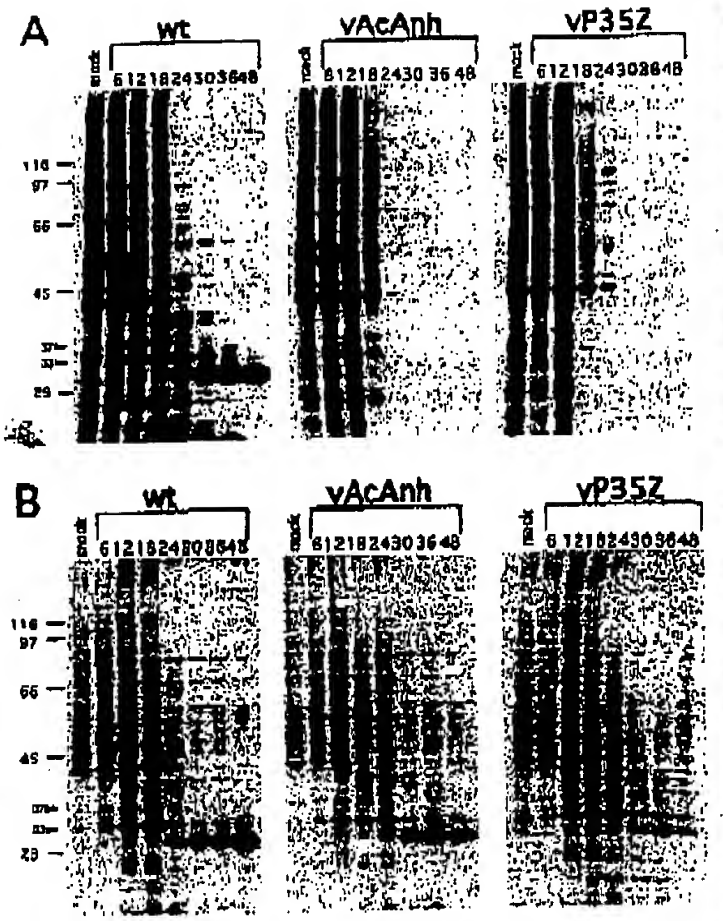

FIG. 1. Kinetics of protein synthesis in SF-21 (A) and TN-368 (B) cells infected with wt Ac*M*NPV, vAcAnh, or vP35Z. Cells were either mock infected or infected with the viruses shown, and proteins were pulse-labeled with [^{35}S]methionine for 1 h at the times indicated (hours p.i.). The positions of size markers are shown at the left in kilodaltons, and the positions of the 37K and 33K polypeptides discussed in the text are also indicated by arrowheads at the left.

infected with vAcAnh, vP35Z, or wt were similar in both the timing of appearance and the intensity of virally induced proteins as well as in the shutoff of host proteins (Fig. 1B). The only slight differences detected were a delay in the shutoff of the 33K virally induced early protein discussed above in *p35* mutant-infected lysates compared with wt-infected cells (compare the 24-h lanes) and low levels of polyhedrin in vP35Z-infected lysates compared with wt- and vAcAnh-infected lysates (compare the 24- through 48-h lanes). Both vP35Z and a revertant virus containing a wt *p35* gene (vP35ZRS; see below) were also somewhat defective in production of occlusion bodies in TN-368 cells (5), presumably because of an unidentified second-site mutation affecting polyhedrin synthesis. Comparison of mutant- and wt-infected proteins failed to reveal either a candidate P35 polypeptide or a P35–β-galactosidase fusion protein in the vP35Z-infected samples. Overall, these protein synthesis results are consistent with those reported by Hershberger et al. (16).

Analysis of steady-state levels of selected viral transcripts. In an effort to further characterize viral gene expression in SF-21 and TN-368 cells infected with *p35* mutant viruses, we examined the levels of transcripts from three viral genes in the three main transcriptional classes of Ac*M*NPV genes:

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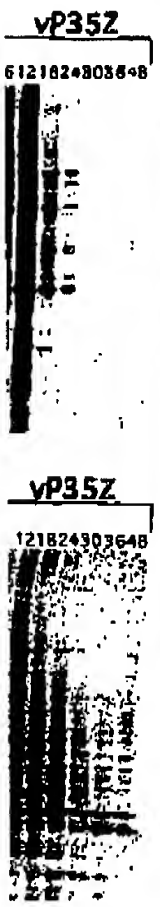

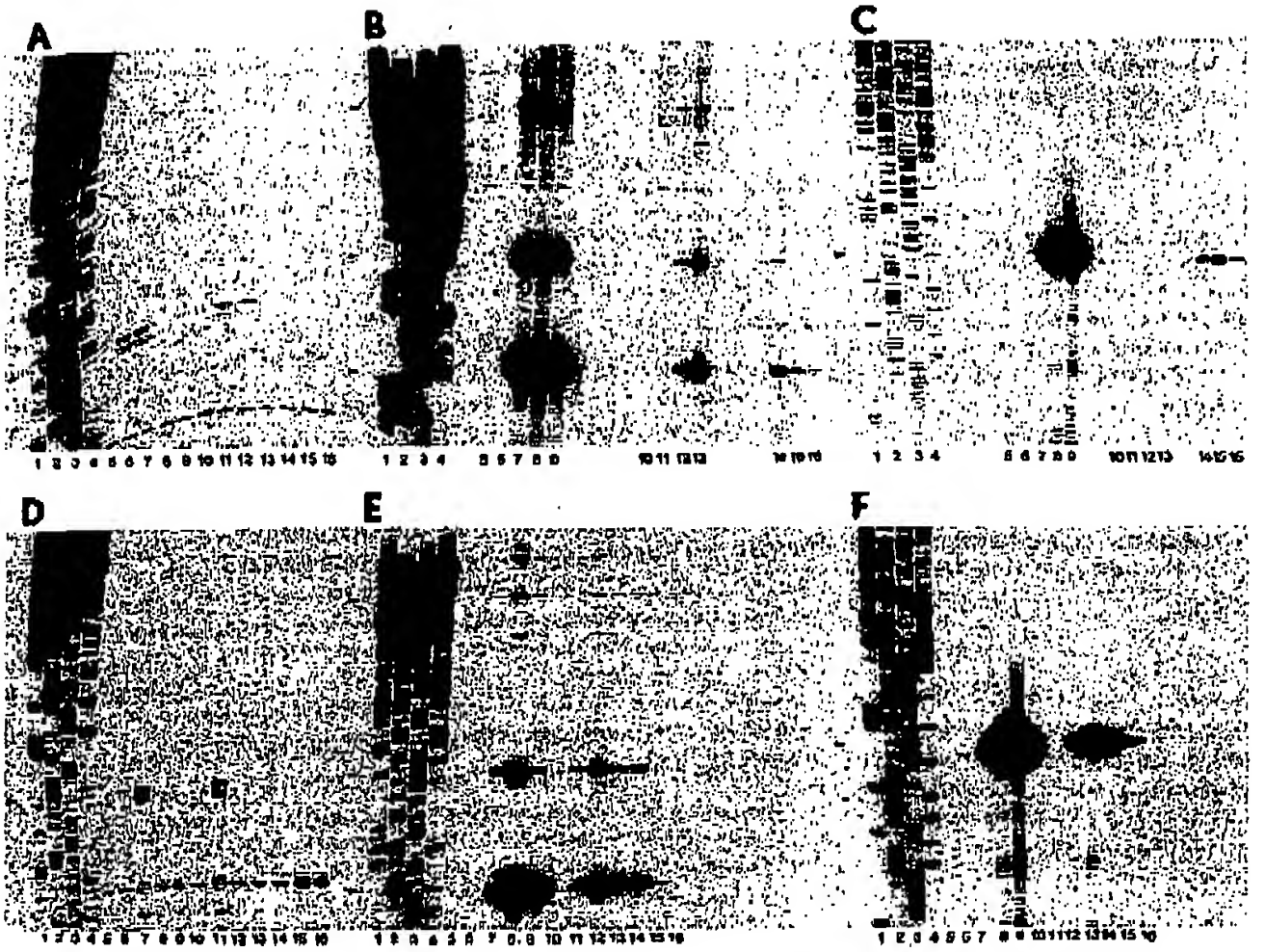

FIG. 2. Steady-state levels of selected viral transcripts during infection of SF-21 (A to C) and TN-368 (D to F) cells with wt AcMNPV or vP35Z. Total RNA was harvested from cells at various times p.i. and analyzed by primer extension, using oligonucleotides complementary to the early *egt* (A and D), the late *vp39* (B and E), or the very late *polh* (C and F) gene. Lanes: 1 to 4, G, A, T, and C lanes of dideoxynucleotide sequencing reactions using the same labeled oligonucleotide primers; 5, RNA from mock-infected cells; 6 to 9, RNA from 6-, 12-, 24-, and 48-h-p.i. cells infected with wt; 10 to 13, RNA from 6-, 12-, 24-, and 48-h-p.i. cells infected with vP35Z; 14 to 16, 1:5-, 1:25-, and 1:125-fold dilutions of the RNA in lane 6 (*egt*) or lane 8 (*vp39* and *polh*) (lanes 14 and 15 are reversed in panel C). Arrowheads at the left indicate the expected positions of the extension products for each oligonucleotide primer.

early, late, and very late. For this, we chose the AcMNPV genes *egt* (the gene encoding ecdysteroid UDP-glucosyltransferase [34]), *vp39* (the gene encoding the major capsid protein [39]), and *polh* (the gene encoding polyhedrin [18]), respectively. The level of transcripts from each of these three genes was examined by primer extension in both wt- and vP35Z-infected SF-21 (Fig. 2A to C) and TN-368 (Fig. 2D to F) cells. The results obtained with wt AcMNPV corresponded well with those previously published for those genes in both the temporal accumulation of the transcripts and location of the 5′ termini (32, 34, 39).

Transcripts from the early gene *egt* were delayed in their appearance and disappearance in vP35Z-infected SF-21 cells compared to wt-infected cells (Fig. 2A). Whereas the transcripts were detected at 6 and 12 h p.i. in wt-infected cells, they were not detected until 12 h p.i. in vP35Z-infected cells and were also present at 24 h p.i. This delay in accumulation of the *egt* transcript is consistent with the delay seen in the appearance of early proteins in SF-21 cells (Fig. 1A). Similar results were obtained (5) with use of an oligonucleotide primer specific for a different early viral gene, *pcna* (proliferating cell nuclear antigen) (7).

The accumulation of transcripts from the late *vp39* gene was also delayed in vP35Z-infected SF-21 cells (Fig. 2B). *vp39* transcripts were first detected in vP35Z-infected cells at 24 h p.i. and reached maximum levels at 48 h p.i., whereas in wt-infected cells, *vp39* transcripts were first detected at 12 h p.i. and reached maximum levels at 24 h p.i. (Fig. 2B). In addition, the maximum accumulation of *vp39* transcripts appeared to be approximately fivefold lower in vP35Z-infected SF-21 cells than in wt-infected cells.

Levels of transcripts from the very late *polh* gene were extremely low in vP35Z-infected SF-21 cells (Fig. 2C) and were barely detectable even upon overexposure of the autoradiograph (data not shown), whereas *polh* transcripts were first detected at 24 h p.i. in wt-infected cells and reached a maximum at 48 h p.i. (Fig. 2C).

In TN-368 cells (Fig. 2D to F), the levels of accumulation of *egt*, *vp39*, and *polh* transcripts were similar during infection with vP35Z or wt. Transcripts of *egt* were first detected at 6 h p.i., with maximum levels at 12 h p.i. in both vP35Z- and wt-infected TN-368 cells (Fig. 2D; a band corresponding to the *egt* transcript was clearly visible in lane 10 on the original autoradiograph), while *vp39* transcripts were detected at 12, 24 and 48 h p.i., with maximum levels at 24 h p.i. (Fig. 2E), and *polh* transcripts were detected at 24 and 48

h p.i., with maximum levels at 48 h p.i. (Fig. 2F). The maximum levels of *polh* transcript were approximately five-fold lower in vP35Z-infected TN-368 cells than in wt-infected cells, corresponding with lower levels of polyhedrin protein observed in Fig. 1B.

Yields of BV from *p35* mutant-infected cells. Premature death of the host cell by apoptosis would be expected to interfere with the replication of the virus. Having observed altered viral transcription and protein synthesis in SF-21 cells, we next determined the effect of apoptosis on the yield of progeny BV. The levels of progeny BV resulting from a single burst of replication (each cell being infected at the outset of the experiment) in SF-21 and TN-368 cells was determined for wt Ac*M*NPV, vAcAnh and its revertant, vAnhHK5, and vP35Z and its revertant, vP35ZRS, by plaque assay using TN-368 cells (Fig. 3). The revertant viruses vAnhHK5 and vP35ZRS were constructed by replacement of the mutant copy of *p35* in vAcAnh and vP35Z with a wt copy of *p35* (see Materials and Methods).

The release of BV was similar in SF-21 cells infected with each of the five viruses through 12 h p.i. (Fig. 3A), which was approximately the time when apoptosis was beginning to become evident morphologically. A 50- to 100-fold difference in progeny BV yield was seen between the *p35* mutants and their revertants (Fig. 3A) at 24 h p.i. At 48 h p.i., the differences were less dramatic (20- to 40-fold) despite the fact that protein synthesis was observed to decline after 24 h p.i. in SF-21 cells infected with the *p35* mutants (Fig. 1A) and the majority of the cells were apoptotic by this time (3). It may be that lysis of the apoptotic bodies between 24 and 48 h p.i. resulted in the release of additional intracellular virus which had been assembled prior to 24 h p.i. The revertants and wt produced comparable levels of progeny BV in the two cell lines (Fig. 3).

The levels of progeny BV obtained from infection of TN-368 cells with the five viruses were essentially identical at all time points examined (Fig. 3B). This result, in addition to the protein synthesis and RNA accumulation data presented above, indicates that *p35* is not required for normal replication in TN-368 cells.

These results are consistent with those reported by Hershberger et al., who examined BV yields at 48 h p.i. (16). Although they observed a much greater difference in levels of progeny BV obtained from SF-21 cells infected with a *p35* mutant and wt Ac*M*NPV (16), this larger difference was almost certainly due to the lower MOI that they used, which would allow for multiple rounds of replication and amplification of any existing differences between the mutants and wt.

Infectivity of *p35* mutants in insect larvae. Although the lack of the *p35* gene results in apoptosis and thereby a reduction in Ac*M*NPV replication in SF-21 cells, it was important to determine whether this effect also occurred in the whole organism or whether it was simply a cell line-specific effect. Since apoptosis reduces the yield of progeny BV from SF-21 cells, one prediction of the effect of an apoptotic response on virus infection in vivo would be an increase in the amount of virus required to initiate infection. We thus determined the approximate dosages of BV required for 50% lethality (LD_{50}s) of wt Ac*M*NPV, the two *p35* mutants vAcAnh and vP35Z, and their revertants vAnhHK5 and vP35ZRS, in *S. frugiperda* and *T. ni* larvae. As seen in Fig. 4A, the LD_{50} in fifth-instar *S. frugiperda* larvae was approximately 1,000-fold higher for the *p35* mutants than for wt Ac*M*NPV or their revertants. Wild-type Ac*M*NPV and both of the revertants had LD_{50}s between 10 and 100 PFU per larva, which were similar to previously reported results (9). Since the LD_{50}s of the revertants were similar to that of wt Ac*M*NPV, any differences seen between the two mutants and wt were due to the presence of the *p35* gene.

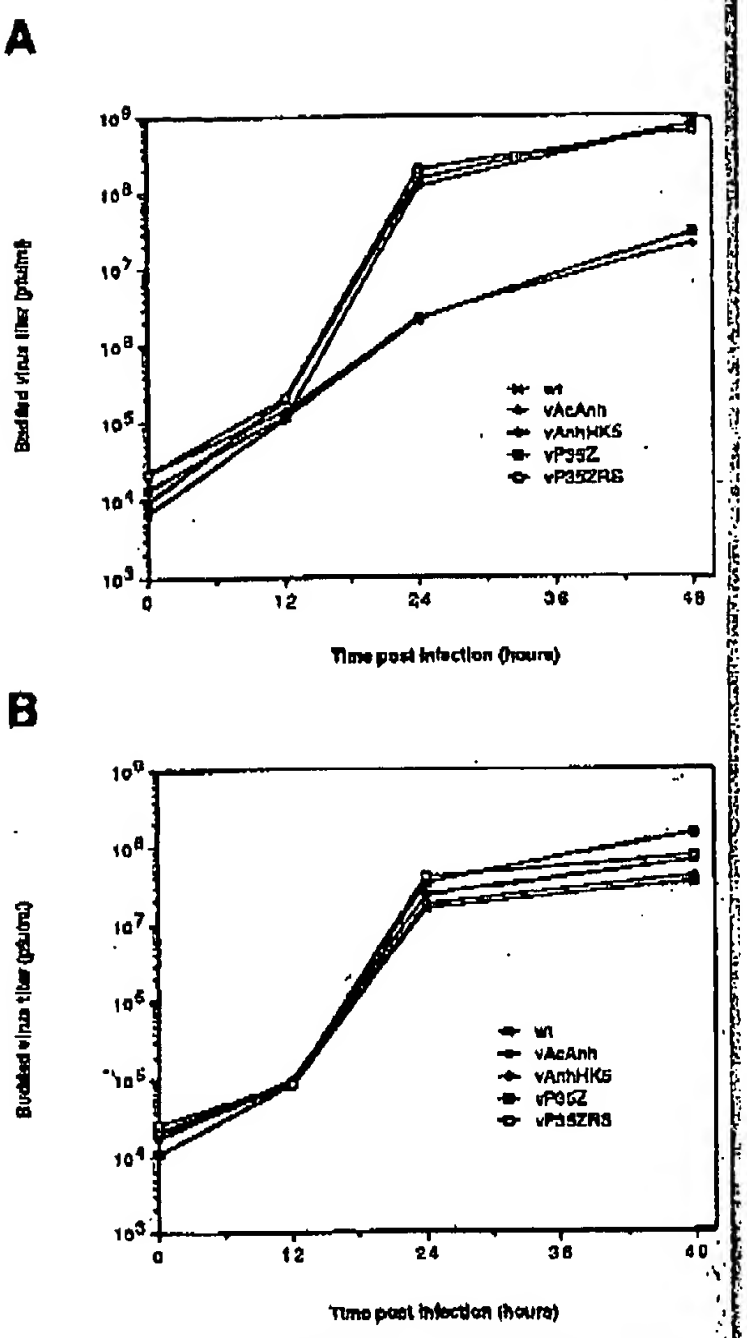

FIG. 3. Yield of progeny BV from SF-21 (A) and TN-368 (B) cells infected with wt Ac*M*NPV, the *p35* mutant vAcAnh or vP35Z, or the revertant virus vAnhHK5 or vP35ZRS. Cell monolayers were infected at an MOI of 20 PFU per cell, and samples of the culture supernatant were harvested at 0, 12, 24, or 48 h p.i. and titered by plaque assay using TN-368 cells. The results shown represent the averages of two independent experiments.

In fourth-instar *T. ni*, the LD_{50}s of all five of the viruses were between 1 and 10 PFU per larva (Fig. 4B). The lower LD_{50} of the budded form of Ac*M*NPV in *T. ni* compared with *S. frugiperda* has been reported previously (9). There were no significant differences among the LD_{50}s of wt, the *p35* mutants, or the revertant viruses in *T. ni* larvae. Thus, the

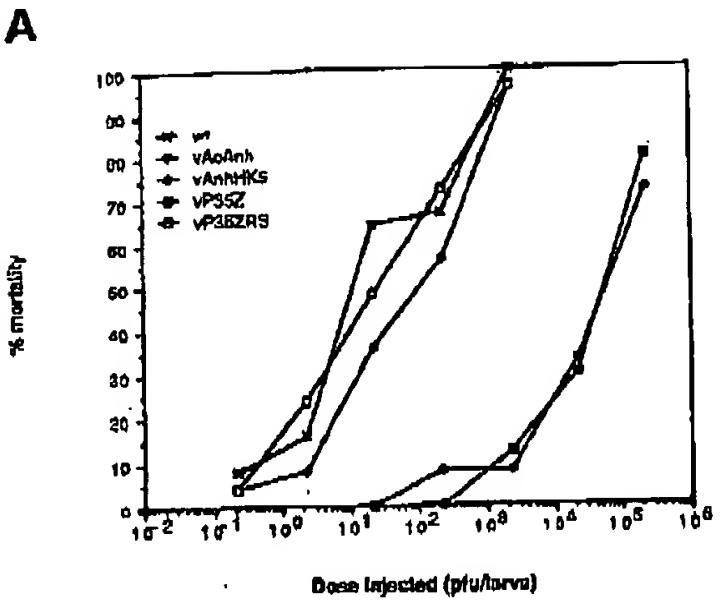

FIG. 4. Mortality due to virus infection in *S. frugiperda* (A) and *T. ni* (B) larvae injected with various doses of wt AcMNPV, vP35Z, vAcAnh, vAnhHK5, or vP35ZRS. Twenty-five larvae were injected per dose; percent mortality was calculated as the number of dead larvae (minus larvae which died as a result of the injection procedure) divided by the number of larvae which survived the injection procedure (at least 20, except for the highest doses of wt and vAcAnh in *T. ni*, which were 19 and 18, respectively). No mortality was observed in mock-infected larvae.

differential response of SF-21 and TN-368 cells to infection with the *p35* mutants was also observed at the organismal level.

Another prediction arising from an apoptotic response in larvae would be a large reduction in the yield of the occluded form of the virus (OV), since *p35* mutant-infected SF-21 cells do not produce any occlusion bodies (3). We therefore determined the yield of OV from the larvae which died from viral infection at the highest doses injected. *S. frugiperda* larvae infected with vAcAnh produced approximately 900-fold less OV than did larvae infected with the revertant virus vAnhHK5, whereas approximately 4-fold less OV was produced in *T. ni* larvae infected with vAcAnh than in larvae infected with vAnhHK5 (Table 1).

TABLE 1. OV yields from *S. frugiperda* and *T. ni* larvae[a] infected with wt AcMNPV, vAcAnh, or vAnhHK5

Host	Virus	OV/larva[b] ± SE[c]
S. frugiperda	wt	$6.5 \times 10^8 \pm 6 \times 10^7$
	vAcAnh	$1.4 \times 10^6 \pm 1 \times 10^5$
	vAnhHK5	$1.2 \times 10^9 \pm 2 \times 10^7$
T. ni	wt	$1.8 \times 10^8 \pm 2 \times 10^7$
	vAcAnh	$2.7 \times 10^7 \pm 9 \times 10^5$
	vAnhHK5	$1.0 \times 10^8 \pm 3 \times 10^6$

[a] The larvae used were those which were injected with the highest doses of BV in Fig. 4 and which died from virus infection.
[b] Larvae were pooled and homogenized in a total volume of buffer equivalent to the number of larvae per sample (in milliliters).
[c] Based on two independent counts of the same sample.

A significant difference was also seen in the melting and liquefaction of the cadavers between the larvae injected with the two *p35* mutants versus wt or the two revertants. Late in wt AcMNPV infection, the infected cadaver normally breaks down as the muscles and other tissues liquefy, and the contents of the dead larva (mainly OV) are released into the environment. Both *S. frugiperda* and *T. ni* larvae infected with vP35Z or vAcAnh did not melt; although the insects became flaccid, the cuticle did not rupture. Many of the dead larvae infected with the *p35* mutants also did not melanize. Melting was normal in larvae infected with wt or the revertant viruses.

DISCUSSION

Although the requirement for the AcMNPV *p35* gene product in blocking programmed cell death in the SF-21 cell line has been demonstrated previously (3, 16), it was not known whether *p35* was required only in cell culture or whether it also plays a similar role in the natural host. Our results show that mutation of *p35* results in greatly reduced levels of AcMNPV replication and infectivity in both SF-21 cells and *S. frugiperda* larvae but not in TN-368 cells or *T. ni* larvae.

T. ni and *S. frugiperda* larvae appear to have little or no effective defense against infection by wt AcMNPV via injection of BV into the hemocoel. Very few infectious virus particles (PFU determined by plaque assay in tissue culture) were required to establish a lethal infection in larvae of these two species, with the LD_{50} for wt AcMNPV being between approximately 1 and 10 PFU per larva in *T. ni* and between 10 and 100 PFU per larva in *S. frugiperda*. The higher LD_{50} in *S. frugiperda* was probably due not to an organismal defense system but to the intrinsically lower infectivity of AcMNPV in *S. frugiperda* cells; titers of wt AcMNPV determined in *S. frugiperda* cell lines are consistently 5- to 10-fold lower than titers determined in *T. ni* cell lines (5, 9, 26). Thus, any host defense to virus invasion which may exist in these insects appears to be inadequate in preventing lethal infection by wt AcMNPV.

Inactivation of the AcMNPV *p35* gene resulted in an increase in LD_{50} of 3 orders of magnitude in *S. frugiperda* larvae. Since infection with *p35* mutants results in apoptosis in SF-21 cells, the most straightforward interpretation of this result is that an apoptotic response by cells in the *S. frugiperda* larvae decreased the ability of the *p35* mutant viruses to establish a lethal infection. The observation that

the LD_{50}s of the *p35* mutants were equivalent to that of wt in *T. ni* larvae served as a control, since the *p35* mutants did not cause apoptosis in TN-368 cells. At the current time, however, we do not have supporting data to demonstrate that apoptosis is occurring in vivo. Apoptotic cells were not consistently observed in the hemolymph of *p35* mutant-infected insects (5), although this may have been due to a combination of the asynchronous nature of in vivo infections and rapid phagocytosis of apoptotic cells by hemocytes.

Regardless of the mechanism by which *p35* functions to increase infectivity in *S. frugiperda*, its presence would be expected to provide a clear evolutionary advantage for AcMNPV replication in this species. *p35* therefore effectively constitutes a host range determinant in AcMNPV, and acquiring *p35* has probably allowed the virus to expand its practical host range to other species, including *S. frugiperda*.

Although the LD_{50} for the *p35* mutants was approximately 1,000-fold higher than that of wt in *S. frugiperda* larvae, the decrease in BV yield from SF-21 cells infected with *p35* mutants was only 50- to 100-fold compared with the wt level. This difference might be explained by several factors, including the following: (i) the high MOI used to infect SF-21 cells may have accelerated the course of infection and increased the amount of BV produced before apoptosis was fully implemented, and (ii) during in vivo infection, phagocytic cells may recognize apoptotic cells and engulf them before large amounts of BV are released, resulting in inactivation of intracellular virus (6).

The differential response to *p35* mutant infection in *S. frugiperda* and *T. ni* appears to be largely species specific; species-specific effects were observed in both in vivo infectivity and OV production. However, the lack of melting in both *S. frugiperda* and *T. ni* larvae infected with *p35* mutants indicates that infection of both species is affected to some degree by inactivation of *p35*. The physiological basis for larval melting is unknown, and the connection between melting and apoptosis, if one exists, is far from clear at this time. Since a normal melting phenotype was restored in the *p35* revertants vAnhHK5 and vP35ZRS, it is unlikely that a second-site mutation was responsible for the lack of melting. It is possible that melting requires the infection of a specific tissue of the larva or that it requires the production of a melting factor. If P35 is required for either of these possibilities, then melting would not occur in *p35* mutant-infected larvae of either species.

It is possible that the species-specific effects of *p35* mutation lie in a difference in the proportions of types of cells or tissues that are sensitive to apoptosis in the two species. This explanation is supported by the observation that a low level of OV was produced in *S. frugiperda* larvae infected with vAcAnh, indicating that some tissues of this species allowed completion of the viral replication cycle and consequent production of OV. In addition, three- to fourfold less OV was produced in *T. ni* larvae infected with vAcAnh than in larvae infected with vAnhHK5, suggesting that there may be a small proportion of tissues which undergo apoptosis in this species as well. Although we have not extensively studied the effect of larva-to-larva variability on OV yield, initial experiments on individual *S. frugiperda* larvae infected with *p35* mutant viruses indicated that a low level of OV was produced in each larva (5).

Our results contrast with those of Kamita et al. (21), who studied the in vivo effect of mutating the BmNPV *p35* gene by injecting *B. mori* larvae with BV. No differences were observed between wt BmNPV and the *p35* mutant in terms of infectivity, OV yield, or survival time. However, these results are difficult to interpret given the mixed in vitro phenotype of their mutant virus (21). Furthermore, only very high dosages of BV (5×10^5 PFU per larva) were used (21), so that any difference in LD_{50} between their *p35* mutant and wt BmNPV may not have been detectable.

Analysis of gene expression in SF-21 cells infected with *p35* mutants revealed a delay in early gene expression, a failure of the mutant to shut off host protein synthesis in a timely fashion, a defect in the ability of the mutants to initiate late protein synthesis, and an eventual decline in total protein synthesis in apoptotic cells. The pattern of protein synthesis in these cells indicated that the only viral proteins synthesized were primarily those of the early class and that their appearance was delayed compared with wt infection. Similar protein synthesis patterns were obtained by Hershberger et al. (16), and a delay in the synthesis of several early viral proteins is also apparent in their data. Analysis of the transcription of selected viral genes revealed a delay in both the early (*egt*) and late (*vp39*) transcriptional phases and a significant reduction in the levels of late and very late (*polh*) gene transcription. The virtual lack of late protein synthesis despite the presence of some level of late RNAs may be related to the observation that total protein synthesis is shut off by 36 h p.i., a time at which most cells are apoptotic. If apoptosis is accompanied by a shutoff in protein synthesis, then cells which initiate late gene transcription might synthesize some late viral RNAs but little or no synthesis of late proteins would be observed. Similar results have been reported with a herpes simplex virus type 1 mutant which induces programmed cell death in neuroblastoma cells (2). It remains to be determined whether the shutoff of protein synthesis is a general phenomenon of apoptotic cells or a characteristic only of virally infected apoptotic cells.

The data show that P35 accelerates AcMNPV infection in SF-21 cells, and we currently favor the hypothesis that P35 accelerates and/or intensifies, directly or indirectly, the expression of other viral genes, including at least one which is directly responsible for blocking apoptosis. The delay or reduction in the level of early gene expression could provide enough time for SF-21 cells to mount an irreversible apoptotic defense to *p35* mutant infection. In contrast, *p35* expression in wt-infected cells would allow timely and sufficient expression of early and late genes so that the apoptotic program, even if transiently initiated, can be blocked. Hershberger et al. (16) have suggested that P35 may be involved in the general infectivity of AcMNPV in SF-21 cells; this would also be consistent with a role for P35 in accelerating the infection process, particularly if timely and effective expression of early viral genes is critical to the viral invasion strategy. We suspect that the observed delay in early viral gene expression is a particularly critical feature of the *p35* mutant phenotype because cells infected with a mutant of AcMNPV carrying a null mutation in the viral *pcna*-homologous gene show a delay specifically in DNA replication and the initiation of late gene expression, but these cells do not undergo apoptosis (7, 33).

Further, we prefer the view that P35 or the factor which it regulates acts directly in blocking apoptosis in SF-21 cells because apoptosis appears to be initiated at the same time in both wt- and *p35* mutant-infected SF-21 cells; cell surface blebbing, a morphological change evident during the early stages of apoptosis, is observed at the same time (approximately 12 h p.i.) in both cases (3). In wt-infected cells, this blebbing is transient and subsides during the later stages of

infection, whereas blebbing intensifies as *p35* mutant infections proceed. If apoptosis is indeed initiated in both wt- and *p35* mutant-infected SF-21 cells, then P35 or the antiapoptotic viral gene product(s) stimulated by P35 function is likely to act by directly blocking the apoptotic program at some crucial point.

The hypothesis that timely expression of at least one early viral gene product can block apoptosis in *S. frugiperda* cells is strongly supported by our previous observation that wt AcMNPV, but not vAcAnh, can prevent apoptosis triggered by actinomycin D treatment if the actinomycin D is added at 5 h p.i. or later, but wt infection cannot block apoptosis if the actinomycin D is added prior to 5 hours p.i. (8). These experiments do not distinguish whether P35 acts directly or activates the expression of other viral gene products to block actinomycin D-induced apoptosis.

The fact that the CpGV *iap* gene, a zinc finger-like gene which is distinct from *p35*, can functionally substitute for *p35* in blocking *p35* mutant-induced or actinomycin D-induced apoptosis in SF-21 cells (8) provides additional perspective to the question of P35 function. Since the CpGV *iap* gene has a homolog (Ac-*iap*) in the AcMNPV genome and Ac-*iap* is unable to functionally substitute for the AcMNPV *p35* gene in blocking apoptosis (8), it is possible that P35 regulates Ac-IAP activity in some way, either by direct interaction with Ac-IAP at the protein level or by stimulation of Ac-*iap* expression. In either case, a central (but not necessarily direct) role for *iap* genes in blocking apoptosis in SF-21 cells is indicated. Since the predicted products of these genes have zinc finger-like motifs, they are likely to act at the level of gene regulation, but whether they act directly to regulate the cellular genes involved in implementing the apoptosis program or whether they act to regulate other viral genes remains to be determined.

The observation that viral protein synthesis and transcription were normal in *p35* mutant-infected TN-368 cells leads to the question of what specific role P35 plays in SF-21 cells or, alternately, why P35 is not required in TN-368 cells. There are at least four possibilities why P35 function may be unnecessary for normal replication in *T. ni* cells. (i) AcMNPV may not trigger apoptosis in this species so that P35 or P35-regulated factors which are required to block the programmed response are not necessary. Support for this view comes from the observation that the cell surface blebbing indicative of early apoptosis is not observed even transiently in TN-368 infections (3). However, apoptosis is a common response to infection by large DNA-containing viruses (4) and appears to be an important organismal defense to viral invasion, so it would be unclear why *T. ni* cells lack this response. (ii) There may be a second (redundant) pathway by which AcMNPV is able to block apoptosis in *T. ni* cells but which is ineffective in *S. frugiperda* cells. If this pathway worked so as to block even the initiation of apoptosis, then neither initial cell surface blebbing nor eventual apoptosis would be observed in *T. ni* cells. However, this would not explain why the presence of intact *p35* accelerates infection in SF-21 cells but not TN-368 cells. (iii) The regulation of early gene transcription in *T. ni* and *S. frugiperda* cells may differ so significantly that P35 may be required for timely and intense early gene transcription only in *S. frugiperda* cells. Support for this view comes from the observation that the regulation of transcription of at least one early AcMNPV gene differs substantially in TN-368 and SF-21 cells; the primary 1.7-kb transcript of the DA26 ORF of AcMNPV is synthesized earlier in TN-368 cells than in SF-21 cells (36). (iv) The *p35*-regulated viral gene product(s) which blocks apoptosis may interact more effectively in TN-368 cells than in SF-21 cells and is therefore required in less abundance to be equally effective in blocking apoptosis. These four possibilities may not be mutually exclusive, and one or some combination of them may be involved.

We conclude that cellular apoptosis can have a drastic effect on the outcome of AcMNPV infection in the insect host. Our results indicate that apoptosis can play a major role in insect immunity to virus infection and that the ability to block apoptosis is a determining factor in baculovirus host range. Our data also show that P35 accelerates the infection of SF-21 cells and suggest a role for P35 in accelerating or intensifying the synthesis of early viral gene products. One or more of these viral gene products may be directly responsible for blocking the cellular apoptosis program.

ACKNOWLEDGMENTS

We thank S. Hilliard for technical support, M. Robson for preparing insect diet, and B. Ribeiro for providing TN-368 cells. We also gratefully acknowledge D. Perkins (USDA-ARS, Tifton, Ga.) and B. Gray (Abbott Laboratories, Chicago, Ill.) for providing insect eggs.

R.J.C. received support from NIH training grant GM07103 during a portion of the time this work was done. This research was supported in part by Public Health Service grant AI29719 from the National Institute of Allergy and Infectious Diseases to L.K.M.

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JOURNAL OF VIROLOGY, Sept. 1992, p. 5525–5533
0022-538X/92/095525-09$02.00/0

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Site-Specific Mutagenesis of the 35-Kilodalton Protein Gene Encoded by *Autographa californica* Nuclear Polyhedrosis Virus: Cell Line-Specific Effects on Virus Replication

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Received 13 April 1992/Accepted 17 June 1992

The gene encoding the 35-kDa protein (35K gene) located within the *Eco*RI-S genome fragment of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) is transcribed early in infection. To examine its function(s) with respect to virus multiplication, we introduced specific mutations of this early gene into the Ac*M*NPV genome. In *Spodoptera frugiperda* (SF21) culture, deletion of the 35K gene reduced yields of extracellular, budded virus from 200- to 15,000-fold, depending on input multiplicity. Mutant replication was characterized by dramatically diminished levels of late and very late (occlusion-specific) virus gene expression and premature cell lysis. In contrast, 35K gene inactivation had no effect on virus growth in cultured *Trichoplusia ni* (TN368) cells. Insertion of the 35K gene and its promoter at an alternate site (polyhedrin locus) restored virus replication to wild-type levels in SF21 culture. Subsequent insertion of 4 bp after codon 81 generated a frameshift mutant that exhibited a virus phenotype indistinguishable from that of 35K deletion mutants and demonstrated that the 35K gene product (p35) was required for wild-type replication in SF21 cells. Mutagenesis also indicated that the C terminus of p35, including the last 12 residues, was required for function. In complementation assays, wild-type virus bearing a functional 35K gene allele stimulated all aspects of 35K null mutant replication and suppressed early cell lysis. These findings indicated that p35 is a *trans*-dominant factor that facilitates Ac*M*NPV growth in a cell line-specific manner.

Early genes encoded by *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), the prototype of the subgroup A baculoviruses, are of interest because they influence important regulatory processes of the virus and the host insect during infection. Early Ac*M*NPV genes include those involved in transactivation of virus gene expression (IE0, IE1, and IE-N), virus DNA replication (DNA polymerase, *pcna* [proliferating cell nuclear antigen], and DNA helicase), and suppression of insect metamorphosis (*egt* [ecdysteroid UDPglucosyltransferase]) (2, 4, 12, 17, 22, 27, 34). The expression of early genes from the 128-kb Ac*M*NPV genome requires host RNA polymerase II and initiates prior to virus DNA synthesis. In contrast, expression of late and very late genes depends on a distinct RNA polymerase and DNA replication (for reviews, see references 1, 6, and 28). Late virus genes include those involved in the production of virus progeny that are composed of two infectious forms: enveloped virions that bud from the plasma membrane (budded virus [BV]) and occluded virus particles that accumulate later in the nucleus of the host cell prior to lysis that occurs from 60 to 90 h after infection. Since proper expression of early virus genes is required for subsequent late gene expression, they play a critical role in virus replication and may function as determinants of Ac*M*NPV host range.

We have investigated the role of the early 35-kDa protein gene (35K gene) encoded by the *Eco*RI-S genome fragment of Ac*M*NPV (86.8 to 87.9 map units [m.u.]) in facilitating virus replication in a cell line-specific manner. Our interest in the function and regulation of the 35K gene originated with the finding that the nearby insertion of a host-derived retrotransposon dramatically altered transcription within the region (8, 9, 24). To initiate studies of the possible function(s) of the affected genes, including the 35K gene, we have constructed deletion (null) mutants of Ac*M*NPV and examined the effect of such mutations on virus replication processes. The 35K gene encodes a 35-kDa polypeptide (p35), as demonstrated by in vitro translation of hybrid-selected mRNA from Ac*M*NPV-infected cells (7). Characteristic of early Ac*M*NPV genes, the predominant mRNA (α_1) for p35 is detected within the first hour after infection, accumulates through 6 to 8 h, and declines thereafter (25). Host RNA polymerase II-mediated transcription is controlled by an early virus promoter (composed of a TATA sequence and RNA start site) that is influenced by regulatory motifs located immediately upstream (5, 16, 25). A weaker late-promoter element is located near the early RNA start site (25) and may function to extend 35K gene expression into the later stages of infection.

Recent identification of a spontaneous Ac*M*NPV deletion mutant and subsequent characterization of a site-specific deletion mutant by Clem et al. (3) indicated that loss of sequences within the 35K open reading frame (ORF) causes premature lysis and death of infected host cells. This virus-induced phenomenon was cell line specific, since cultured *Spodoptera frugiperda* (SF21) cells were susceptible to lysis whereas *Trichoplusia ni* (TN368) cells were not. The early lysis of SF21 cells was characterized as that resulting from programmed cell death (apoptosis) on the basis of stereotypic changes in cell morphology and degradation of nuclear DNA into oligonucleosome-sized fragments. This indicated that a function of the 35K gene is to directly or indirectly suppress virus-induced apoptosis (3), a process that may be an antiviral defense mechanism by the host (23, 32, 37). The

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exact role of the 35K gene product (p35) has yet to be determined.

In this report, we describe site-directed mutagenesis of Ac*M*NPV to demonstrate that p35 is required for wild-type levels of virus replication, but only in apoptosis-sensitive cells. 35K null mutants exhibited a dramatic growth disadvantage compared with wild-type virus. Moreover, these mutants exhibited highly restricted levels of late and very late virus gene expression. These defects were consistent with the loss of virus DNA, possibly through the activation of host nucleases during apoptosis. 35K null mutants were also *trans* complemented by a functional 35K allele supplied by coinfection with wild-type virus, demonstrating the dominant nature of this gene. p35 is thus the first Ac*M*NPV gene product identified that provides a selective growth advantage in a cell line-specific manner.

MATERIALS AND METHODS

Viruses and cells. The L-1 strain of Ac*M*NPV (20) and indicated virus mutants were propagated in established *S. frugiperda* IPL-SF21 (35) and *T. ni* TN368 (14) cell lines with TC100 growth medium (GIBCO Laboratories) supplemented with 2.6 mg of tryptose broth per ml and 10% heat-inactivated fetal bovine serum (HyClone Laboratories). At time zero, cell monolayers were inoculated with extracellular BV. After a 1-h adsorption, the residual inoculum was replaced with growth medium. The infected cells were then incubated at 27°C.

Plasmid DNAs and transplacement vectors. A transplacement plasmid was constructed for each Ac*M*NPV recombinant generated. For deletion mutant vΔ35K, the 5.37-kb *Bam*HI-*Sst*I fragment of the Ac*M*NPV genome (84.4 to 88.1 m.u.) was first cloned into vector pUC19, producing plasmid pBB/BSst. Plasmid pΔ35K, in which the 35K ORF was deleted, was generated by *Nru*I and *Spe*I digestion of pBB/BSst, end repair with the Klenow fragment of DNA polymerase I, addition of an 8-bp *Bgl*II linker, and intramolecular ligation. Transplacement plasmids for recombinant viruses in which the 35K gene (or mutations thereof) was inserted at the polyhedrin locus (3.96 m.u.) were constructed from vectors containing the *Escherichia coli lacZ* gene. In brief, a 3.3-kb fragment containing the *lacZ* gene under control of the polyhedrin promoter was inserted into the unique *Xho*I and *Kpn*I sites of pEVooc$^+$/PA (5), generating plasmid pEV-lacZ. The *lacZ* fragment was previously excised from plasmid pPOLY-lacZ in which the *lacZ* gene from pMC-1871 (33) was inserted downstream from the polyhedrin promoter cloned into the pBluescript (KS) vector (Stratagene). Next, the 35K gene under control of its own promoter was inserted into pEV-lacZ. This was accomplished by first constructing plasmid p35K-ORF through insertion of a 1.28-kb *Mlu*I-*Eco*RI fragment (end repaired at the *Mlu*I site), containing the entire 35K ORF and promoter sequences, at *Hin*cII and *Eco*RI sites, respectively, of pBluescript. The 35K gene fragment was then excised by digestion with *Xho*I and *Xba*I and inserted into the corresponding sites of pEV-lacZ to generate plasmid pEV-lacZ/35K$^+$. pEV-lacZ/35K$^{Bcl-/-}$ was constructed by *Bcl*I digestion of p35K-ORF, end repair with the Klenow fragment, blunt-end ligation, and insertion of the altered 35K gene fragment into pEV-lacZ, as described above. pEV-lacZ/35K$^{\Delta Spe}$ was generated by *Spe*I digestion of pEV-lacZ/35K$^+$, followed by end repair with the Klenow fragment and blunt-end ligation.

Recombinant viruses. Standard gene replacement methods (28, 36) were used to construct Ac*M*NPV recombinants. To generate viruses wt/lacZ and vΔ35K, 2×10^6 SF21 cells were transfected with transplacement plasmids pEV-lacZ and pΔ35K, respectively, along with wild-type Ac*M*NPV DNA by using Lipofectin (Bethesda Research Laboratories) as described previously (5). Recombinants vΔ35K/lacZ, vΔ35K/lacZ/35K$^+$, and related viruses lacking the 35K gene in its native position (87 to 88 m.u.) were generated in the same way, except that viral DNA was replaced by an equivalent volume of extracellular vΔ35K virus containing approximately 5×10^5 PFU. The later viruses were identified and plaque purified with TN368 monolayers on the basis of their occlusion-negative phenotype and production of β-galactosidase that was visualized by including 150 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml in the agarose overlay. Deletion mutant vΔ35K was identified on the basis of its occlusion-negative plaque phenotype by using SF21 cells. Proper insertion or deletion of sequences was ascertained by restriction mapping and Southern blot analysis of isolated DNA from each recombinant virus (data not shown). All virus titers were obtained by standard plaque assays with TN368 cells. In the case of *lacZ*-containing viruses, blue plaques were counted 4 days after infection in the presence of X-Gal.

Analysis of virus protein synthesis. At the indicated times after infection, the medium above SF21 or TN368 cell monolayers (10^6 cells per plate) was removed and replaced with phosphate-buffered saline (pH 6.2) (20) containing 200 μCi of Trans^{35}S-Label (1,200 Ci/mmol, methionine ≥70%, cysteine ≤15%; ICN Biomedicals, Inc.) per ml. After a 1-h incubation at 27°C, the cells were dislodged, collected by centrifugation (500 × *g* for 5 min), and lysed with 1% sodium dodecyl sulfate (SDS) and 2.5% β-mercaptoethanol. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (19) and autoradiography.

β-Galactosidase assays. Infected SF21 or TN368 cells were dislodged, collected by centrifugation (500 × *g* for 5 min), and washed with ice-cold phosphate-buffered saline (pH 6.2). The cell pellets were suspended in 0.25 M Tris (pH 8.0) and subjected to three freeze-thaw cycles. Clarified extracts (16,000 × *g*) were assayed immediately for β-galactosidase with the substrate *p*-nitrophenyl-β-D-galactopyranoside essentially as described elsewhere (31). When necessary, extracts were diluted with 0.25 M Tris (pH 8.0) to ensure linearity of the assay.

Dot blot hybridizations. SF21 monolayers (10^6 cells) were harvested 48 h after infection as described above. To isolate total intracellular DNA, the cells were suspended in 10 mM Tris (pH 8.0)–1 mM EDTA and incubated in 0.2% SDS and 0.1 mg of proteinase K per ml for 2 to 4 h at 37°C. Protein and RNA were removed by phenol extraction and treatment with 120 μg of RNase A per ml, respectively. DNA (from 10^6 cells) was heat denatured (10 min, 100°C), snap cooled on ice, and mixed with an equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). With a dot blot apparatus, the entire sample was applied to a Hybond-N (Amersham) nylon membrane and prepared for hybridization as prescribed by the manufacturer. Blotted DNA was hybridized to specific DNA probes at 65°C for 16 h. An [α-^{32}P]dATP-labelled, random-primed probe (Amersham) consisting of a pBluescript plasmid containing only *lacZ* specific sequences was used to monitor intracellular levels of vΔ35K/lacZ virus DNA. After the blots were washed, the extent of probe hybridization was determined by measuring radioactivity associated with individual spots with a Betascope 603 Blot Analyzer. The linearity of the assay was demonstrated by simultaneously including increasing

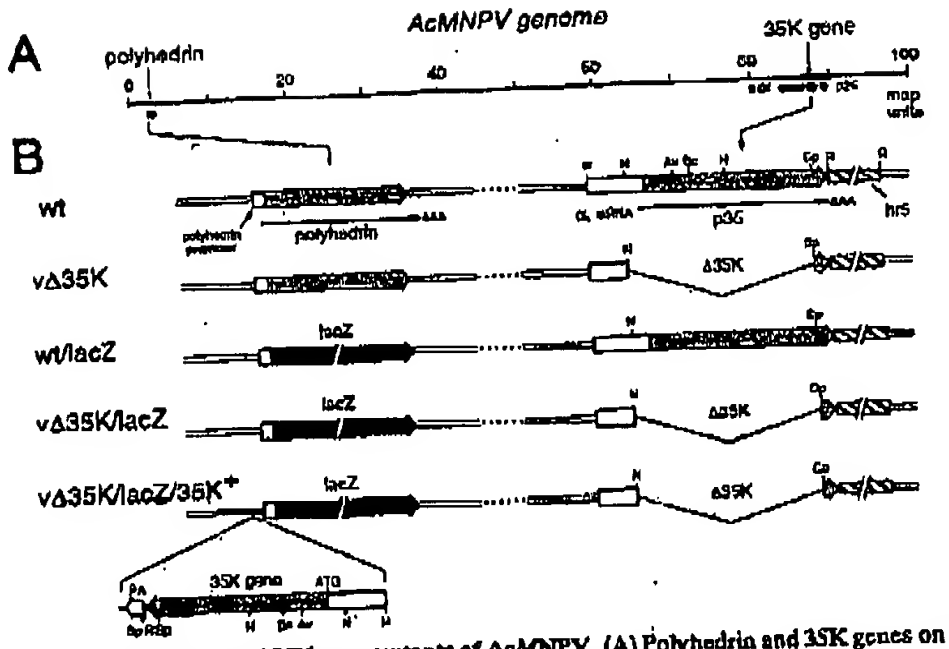


FIG. 1. Genome organization of wild-type and 35K gene mutants of AcMNPV. (A) Polyhedrin and 35K genes on the AcMNPV linear map. Vertical arrows depict the positions of the polyhedrin (4.3 m.u.) and 35K protein (87.4 m.u.) genes; the 35K gene is flanked on either side by the 94K protein and p26 genes. (B) Polyhedrin and p35 locus of wild-type (wt) virus and 35K gene mutants. Virus designations indicate the presence or absence of 35K and *lacZ* genes within the viral genome; protein coding regions are depicted by the shaded arrows. vΔ35K deletion mutants lack the *Nru*I-*Spe*I fragment that includes the promoter and all but 12 codons of the 35K ORF. Viruses wt/lacZ, vΔ35K/lacZ, and vΔ35K/lacZ/35K^{-} contain the *lacZ* gene (under control of the polyhedrin promoter) in place of the structural portion of the polyhedrin gene. Virus vΔ35K/lacZ/35K^{+} contains the 35K gene and its promoter (on a 1.28-kb *Mlu*I-*Eco*RI fragment derived from the *Eco*RI-S region) inserted adjacent (3.96 m.u.) to the *lacZ* gene and upstream from a polyadenylation signal (PA). The α_1 mRNA (1.1 kb) for p35 initiates 26 bp upstream from the predicted initiator codon (ATG) and is polyadenylated 90 bp upstream from the leftmost *Eco*RI site of the hr5 enhancer (striped box). Relevant restriction sites of the 35K gene fragment are indicated. Av, *Ava*II; Be, *Bcl*I; H, *Hin*dIII; M, *Mlu*I; N, *Nru*I; R, *Eco*RI; Sp, *Spe*I.

amounts of membrane-bound *lacZ* plasmid DNA in the hybridization protocol.

RESULTS

Construction of 35K gene null mutations. In preliminary experiments designed to investigate the function(s) of the 35K protein gene, we examined the effect of null mutations on AcMNPV replication by constructing recombinant viruses in which the sequences that encompass the 35K gene were deliberately deleted. To facilitate identification of such mutants, we inserted the *E. coli lacZ* gene into the 35K ORF (between the *Ava*II and *Spe*I sites). This generated a potential fusion protein in which the N-terminal 51 amino acids of p35 were linked to β-galactosidase and simultaneously removed 702 bp of the 35K ORF. When propagated in *S. frugiperda* (SF21) monolayers, the resulting 35K-lacZ virus recombinants exhibited a plaque morphology that was distinct from that of wild-type virus on the basis of smaller size and lack of occlusion bodies; low-level *lacZ* expression was indicated by light-blue plaques in the presence of X-Gal. A similar plaque phenotype was exhibited by an independently constructed AcMNPV mutant, vΔ35K (Fig. 1), in which 950 bp of DNA sequences between the *Nru*I and *Spe*I sites were deleted, thereby removing the 35K promoter and 287 (of 299) codons from the 35K ORF. Both deletion mutants produced unusually low yields of BV in SF21 cultures. In contrast, when propagated in *T. ni* (TN368) cells, mutant virus yields were similar to that of wild-type virus (see below). Moreover, vΔ35K and wild-type virus plaques were indistinguishable, as judged by relative size and yield of occluded virus.

To more accurately examine the growth properties of the 35K null mutants in both lepidopteran cell lines, we constructed a series of 35K ORF mutants in which the structural portion of the polyhedrin gene was replaced by the *lacZ* gene (Fig. 1). Virus wt/lacZ contains the wild-type 35K ORF at its native position (*Eco*RI-S genome fragment), while sequences between the *Nru*I and *Spe*I sites of the 35K ORF were deleted in virus vΔ35/lacZ. Virus vΔ35K/lacZ/35K^{+} contains a single copy of the wild-type 35K ORF and its promoter inserted adjacent to the *lacZ* gene (at the polyhedrin locus). Quantitation of β-galactosidase activity in cells infected by these viruses provided a means to monitor late (occlusion-specific) gene expression. In addition, visualization of blue plaques on TN368 monolayers provided an accurate and reliable measure of infectious BV from 35K null mutants. Since mutant and wild-type viruses exhibited identical growth properties in TN368 cells (see below), all titers in this report are defined as PFU produced on TN368 monolayers.

Sequences that include the 35K ORF are required to maintain wild-type production of BV. To directly compare the growth properties of 35K null mutants, we measured the yields of progeny BV from SF21 and TN368 cultures inoculated with increasing amounts of virus. Since multiple rounds of infection were possible at the lowest multiplicities of infection (MOIs), the yield of BV represented that accumulated over the 48-h period examined and provided an indirect measure of the replication competence of the viruses. In SF21 cells (Fig. 2A), virus vΔ35K/lacZ produced 200- to 15,000-fold less BV than wt/lacZ. The greatest difference (15,000-fold) was observed with an input of 0.1 PFU per cell (Table 1). In contrast to wt/lacZ, the yield of vΔ35K/lacZ increased steadily with the MOI used; a 500-fold increase in MOI (from 0.01 to 5 PFU/cell) yielded a 370-fold

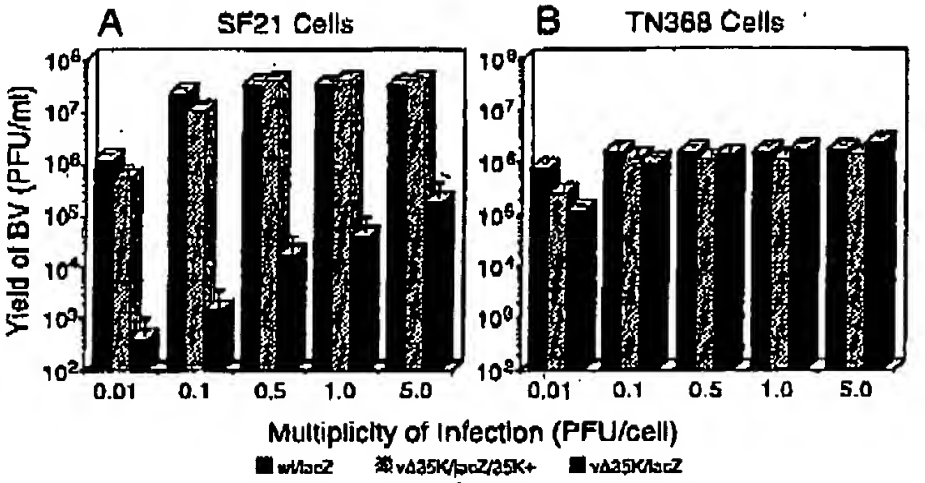


FIG. 2. Production of BV by 35K gene mutants. SF21 (A) and TN368 (B) monolayers (1.8×10^6 cells per plate) were inoculated with viruses wt/lacZ, vΔ35K/lacZ/35K$^+$, and vΔ35K/lacZ at the indicated MOIs (PFU per cell). After a 1-h adsorption, the cells were washed, covered with growth medium (5 ml), and incubated at 27°C. The extracellular medium was harvested 48 h after infection, and the yield of BV was determined by plaque assay with TN368 cells. The plaques were visualized by including X-Gal in the agarose overlay. BV yields are reported as PFU per milliliter with a logarithmic scale; error bars for each value are shown.

increase in BV. Consequently, the ratio of wt/lacZ BV to vΔ35K/lacZ BV decreased with increases in the MOI (Table 1). One-step growth studies indicated that while progeny BV from 35K null mutants appeared at the same time as wild-type BV (24 h after infection), overall levels were reduced throughout infection (0 to 96 h) (21). Visual inspection of vΔ35K/lacZ-infected SF21 cells also indicated that the extent of early cell lysis (characteristic of 35K null mutants) was proportional to the amount of input virus. Lysis resembled that described by Clem et al. (3), including plasma membrane blebbing, progressive cell disintegration, and concomitant degradation of cell DNA into oligonucleosome-sized fragments (data not shown). Lysis of wt/lacZ-infected cells was not observed until 72 to 96 h after infection and did not include cell blebbing.

In contrast, the yield of BV from TN368 cells infected with the 35K null mutant vΔ35K/lacZ was comparable to that of wild-type virus, wt/lacZ (Fig. 2B). Only at the lowest MOI was a significant difference detected; with 0.01 PFU/cell, vΔ35K/lacZ produced approximately sevenfold less BV than wt/lacZ (Table 1). No early cell lysis was detected in TN368 cultures upon inoculation with vΔ35K/lacZ or other 35K null mutants.

BV production was restored to wild-type levels (Fig. 2) when the 35K gene was inserted back into the genome of vΔ35K/lacZ, but at an alternate location (polyhedrin locus [3.96 m.u.]). vΔ35K/lacZ/35K$^+$ yields were comparable to that of wt/lacZ in both cell lines. Only at the lowest MOI (0.01 PFU/cell) did vΔ35K/lacZ/35K$^+$ exhibit a growth disadvantage in SF21 or TN368 cells (respectively, 2.3- and 2.9-fold lower than that of wt/lacZ) (Table 1). The cytopathic effect of vΔ35K/lacZ/35K$^+$ in SF21 cells was also indistinguishable from that of wild-type virus wt/lacZ. Thus, the 1.28-kb *Mlu*I-*Eco*RI fragment that encompasses the 35K ORF and its promoter (Fig. 1) contains the information necessary to restore the wild-type virus phenotype in SF21 culture.

35K null mutants exhibit restricted levels of late virus gene expression. The absence of occluded virus particles in vΔ35K-infected SF21 cells suggested that deletion of the 35K ORF resulted in loss of late virus gene expression. To quantitate this effect, we used the *lacZ* gene under control of the polyhedrin promoter to monitor levels of very late (occlusion-specific) gene expression. In SF21 cells, mutant vΔ35K/lacZ produced only low levels of β-galactosidase at all times examined (Fig. 3). In contrast, β-galactosidase expression in wt/lacZ-infected cells paralleled that of polyhedrin during a wild-type infection (29), reaching a maximum by 48 h and declining thereafter. At peak expression, β-galactosidase levels upon infection with wt/lacZ were 300-fold higher than that of vΔ35K/lacZ. Approximately wild-type levels of β-galactosidase were synthesized in SF21 cells by virus vΔ35K/lacZ/35K$^+$ (Fig. 3), indicating that insertion of the 35K gene and promoter back into the virus genome restored occlusion-specific expression. Identical analyses with TN368 cells indicated that β-galactosidase expression was comparable for all three viruses (Fig. 3). In general, β-galactosidase appeared earlier and reached higher levels in TN368 cells than in SF21 cells.

To examine the effect of 35K null mutations on other

TABLE 1. Comparison of BV yields for wt/lacZ, vΔ35K/lacZ, and vΔ35K/lacZ/35K$^+$

Cell line	Ratio of wt/lacZ to vΔ35K/lacZ[a] at the following MOIs (PFU/cell):					Ratio of wt/lacZ to vΔ35K/lacZ/35K$^+$[b] at the following MOIs (PFU/cell):				
	0.01	0.1	0.5	1.0	5.0	0.01	0.1	0.5	1.0	5.0
SF21	2,900	15,000	2,000	900	200	2.3	2.1	0.9	0.9	0.9
TN368	6.6	1.6	1.2	0.9	0.7	2.9	1.3	1.4	1.4	1.2

[a] The wt:vΔ35K ratio was calculated by dividing the yield (PFU) of wt/lacZ virus by the yield of vΔ35K/lacZ virus obtained at the same MOI.
[b] The wt:vΔ35K/35K$^+$ ratio was calculated by dividing the yield (PFU) of wt/lacZ virus by the yield of vΔ35K/lacZ/35K$^+$ virus obtained at the same MOI.

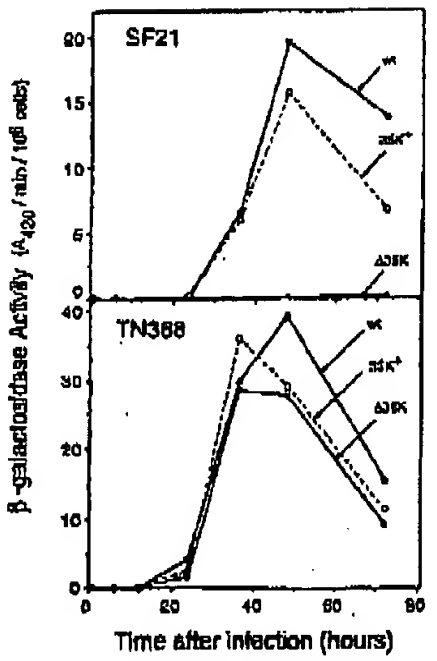


FIG. 3. Comparison of very late, occlusion-specific gene expression by wild-type and 35K null viruses. SF21 and TN368 monolayers (10^6 cells per plate) were inoculated with 5 PFU of recombinant viruses wt/lacZ (wt), vΔ35K/lacZ (Δ35K), and vΔ35K/lacZ/35K$^+$ (35K$^+$) per cell. At the indicated intervals after infection, the cells were harvested, lysed, and assayed for intracellular β-galactosidase. Measured enzyme activity is reported as A_{420} units produced per minute per 10^6 cells. The values shown are the averages of two independent experiments.

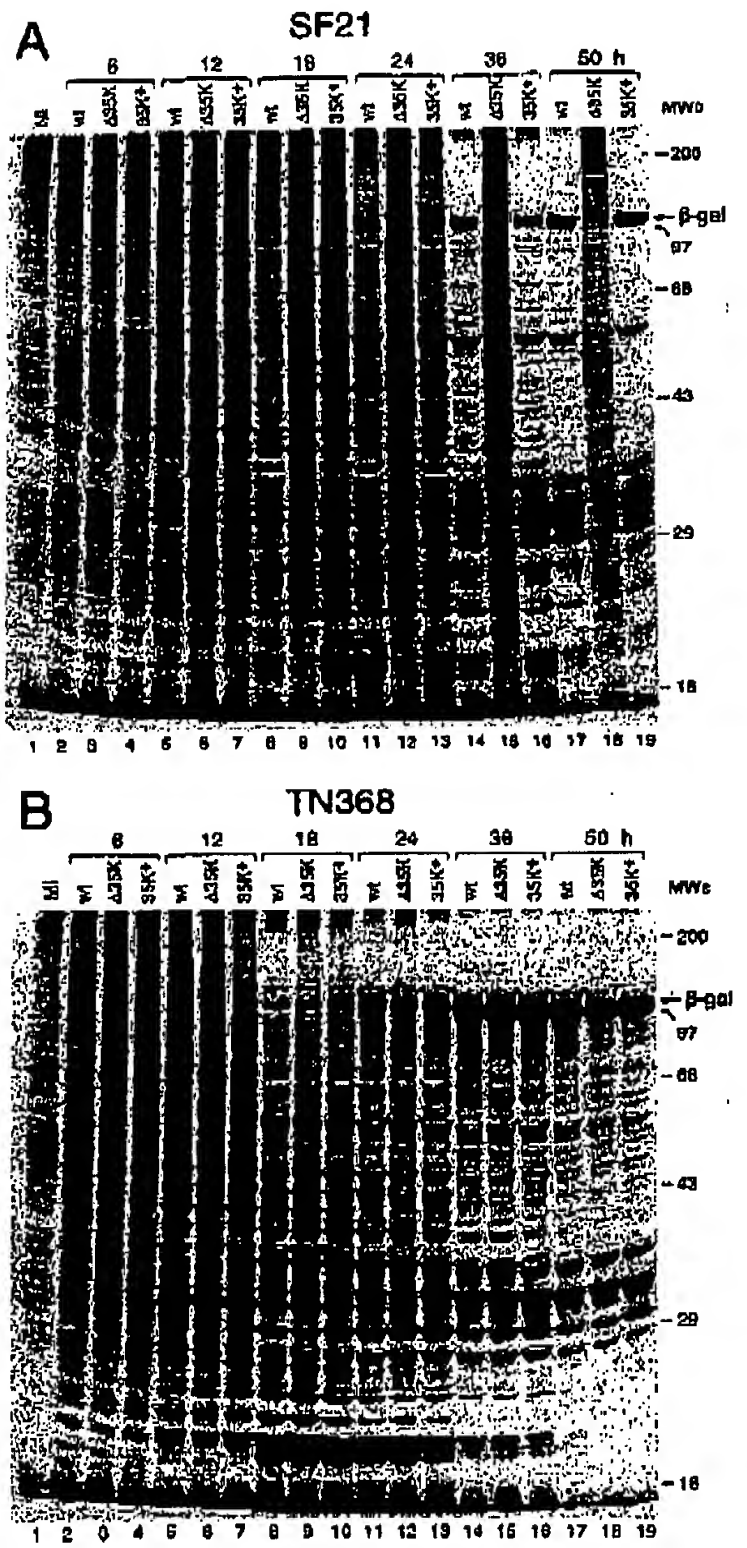


FIG. 4. Comparison of proteins synthesized in cells infected with wild-type and vΔ35K viruses. SF21 (A) and TN368 (B) monolayers were inoculated with 5 PFU of recombinant viruses wt/lacZ (wt), vΔ35K/lacZ (Δ35K), and vΔ35K/lacZ/35K$^+$ (35K$^+$) per cell. At the indicated intervals after infection, the cells were radiolabeled for 1 h with [^{35}S]methionine-cysteine, lysed, and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Lysates prepared from cells radiolabeled after mock-infection (MI) are included (lane 1). The positions of β-galactosidase (β-gal) and molecular weight standards (MWs) are indicated (sizes in kilodaltons).

classes of AcMNPV proteins, we compared protein synthesis throughout infection in both cell lines. SF21 cells infected with viruses wt/lacZ and vΔ35K/lacZ/35K$^+$ exhibited similar protein patterns that were typical of a wild-type virus infection (Fig. 4A); this included the appearance and disappearance of early proteins (6 through 12 h), followed by late proteins (12 through 24 h), and finally very late, occlusion-specific proteins (e.g., β-galactosidase). Although early virus-induced proteins were detected in SF21 cells infected at an identical multiplicity as vΔ35K/lacZ, synthesis of late virus proteins was severely restricted (Fig. 4A). Proteins that normally appeared by 12 h in wild-type infections (during the period of viral DNA replication) were not detected or appeared later and at very low levels. In addition, host protein synthesis continued as late as 50 h after infection; the observed reduction from 36 to 50 h (Fig. 4A) was attributed to vΔ35K/lacZ-induced early cell lysis. Similar analyses (data not shown) indicated that inoculations with higher MOIs of 35K null mutants (including virus vΔ35K [Fig. 1]) caused a more rapid reduction in intracellular protein synthesis; in such cases, little synthesis was detected 36 h after infection. This reduction paralleled a more rapid induction of early cell lysis. Of the cells remaining late in infection, few viral proteins were detected amid the background of host proteins.

The pattern of virus-induced protein synthesis in TN368 cells was nearly identical for each of the three viruses wt/lacZ, vΔ35K/lacZ, and vΔ35K/lacZ/35K$^+$ (Fig. 4B). This included the characteristic reduction in host protein synthesis that began 18 h after infection for each virus. Minor differences were detected in the level of several less prominent early (6 and 12 h) proteins. Direct comparison of the

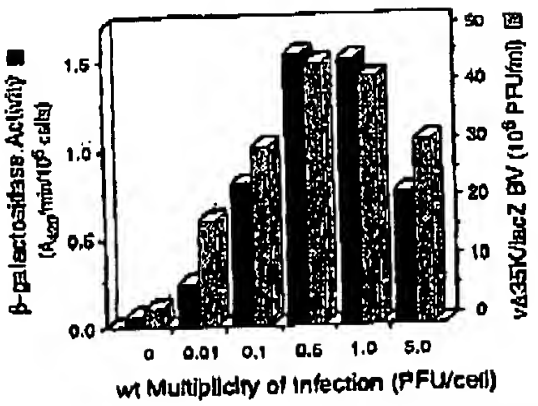


FIG. 5. Effect of wild-type virus on mutant vΔ35K/lacZ late gene expression and BV yields. SF21 monolayers (10^6 cells per plate) were simultaneously inoculated with virus vΔ35K/lacZ (3 PFU/cell) and the indicated MOIs of wild-type (wt) AcMNPV. Cells and extracellular medium were harvested 48 h after infection. Infected-cell extracts were assayed for β-galactosidase; units of activity are as described in the legend to Fig. 3. The yield of vΔ35K/lacZ BV in the extracellular medium was determined by plaque assay on TN368 cells. vΔ35K/lacZ plaques were distinguished from wild-type virus plaques by their blue color (in the presence of X-Gal) and lack of occlusion bodies.

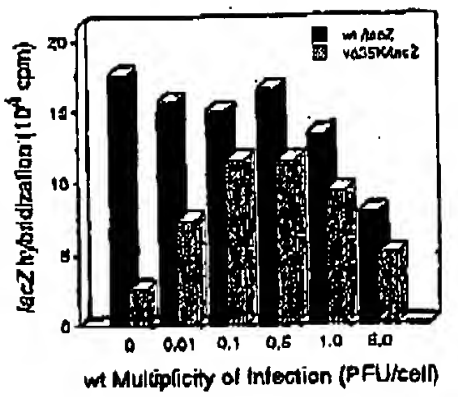


FIG. 6. Effect of wild-type virus on intracellular levels of vΔ35K/lacZ DNA. SF21 monolayers were inoculated with 3 PFU of virus vΔ35K/lacZ or wt/lacZ per cell and the indicated MOI of wild-type (wt) AcMNPV. Intracellular DNA was isolated 48 h after infection. The level of *lacZ*-specific DNA was ascertained by dot blot hybridizations with a radiolabeled DNA probe containing *lacZ* sequences. The extent of hybridization was determined by measuring the radioactivity associated with individual spots and is reported as counts per minute bound.

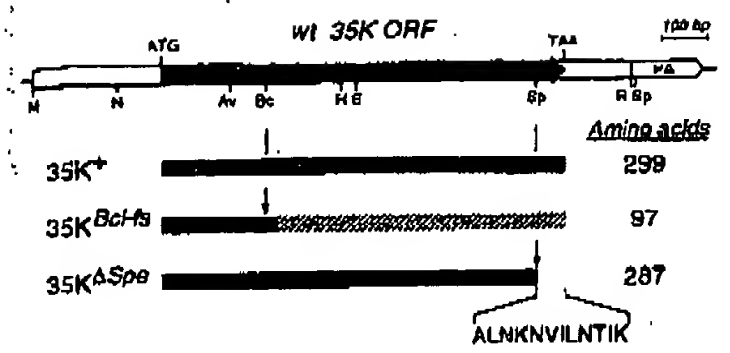
early proteins synthesized in both cell lines (TN368 and SF21) infected with 35K deletion mutants and wild-type virus failed to reveal a 35-kDa polypeptide candidate for the 35K gene product, suggesting that p35 expression is relatively low.

***trans* complementation of 35K null mutants.** To determine whether a functional 35K allele when supplied in *trans* would restore replication of 35K null mutants, we tested the ability of wild-type virus to complement the null mutation. To this end, we monitored BV production and late gene expression in SF21 cells that were inoculated with a fixed MOI of null mutant vΔ35K/lacZ (3 PFU/cell) and increasing amounts of wild-type AcMNPV (Fig. 5). In a way that was indicative of helper virus function, wild-type virus enhanced the production of vΔ35K/lacZ BV; maximum levels of mutant BV were 15-fold higher than that obtained in the absence of wild-type virus. The possibility that this enhancement was due to recombination in which the null mutant acquired a 35K gene was ruled out by screening BV from mixed infections by plaque assay with SF21 cells. By scoring for blue plaques (indicating the rescue of late gene expression), it was determined that less than 5% of the progeny from mixed infections with vΔ35K/lacZ and wild-type virus (0.5 PFU/cell) was composed of recombinant viruses (data not shown). Thus, greater than 95% of the increase in *lacZ*-containing progeny detected (Fig. 5) was represented by mutant vΔ35K/lacZ. Coinfection with wild-type virus also stimulated very late gene expression from vΔ35K/lacZ (Fig. 5); as indicated by β-galactosidase levels, very late gene expression increased as much as 30-fold. At wild-type virus MOIs of greater than 1 PFU/cell, β-galactosidase expression declined. Since a similar reduction was observed in control experiments in which vΔ35K/lacZ was replaced by wt/lacZ (data not shown), we attributed this decline to competition between viruses for limiting host cell factors (e.g., virus receptors or biosynthetic machinery). Lastly, visual inspection of coinoculated cultures also indicated that the extent of early cell lysis induced by vΔ35K/lacZ decreased in proportion to the increase in wild-type virus.

Further support for *trans* complementation by wild-type virus was obtained by measuring intracellular levels of vΔ35K/lacZ-specific DNA in SF21 cells (Fig. 6). As demonstrated by hybridization to a DNA probe complementary to *lacZ* sequences, vΔ35K/lacZ DNA increased by as much as fivefold upon coinfection with wild-type virus (0.1 PFU/cell). An identical increase was observed with a different 35K null mutant (vΔ35K/lacZ/35K^{Bcl-fr}; see below). In contrast, wt/lacZ DNA levels declined as the wild-type virus MOI increased in similar assays (Fig. 6), suggesting again that competition for limiting factors was responsible. Thus, the level of intracellular DNA of 35K null mutants was also affected by wild-type virus containing a functional 35K allele. It remains to be determined whether this increase was due to enhanced viral DNA synthesis by the 35K null mutants or to a reduction in virus DNA loss that resulted from suppression of early cell lysis.

p35 nonsense mutants exhibit the 35K null phenotype. To verify that the 35K null phenotype was due to inactivation (or loss) of the 35K gene product (p35) and not to the removal of *cis*-acting regulatory or replication signals resulting from the deletion of sequences from the mutants described above, we tested the effect of additional 35K ORF mutations on virus multiplication. Since restricted late gene expression was characteristic of the 35K null phenotype, β-galactosidase expression from the polyhedrin promoter was used to monitor virus replication.

First, a nonsense mutation was introduced within the N-terminal third of the 35K ORF through the insertion of 4 nucleotides at the *BclI* site (Fig. 7). This resulted in a frameshift that truncated p35 after 97 amino acid residues without altering sequences downstream. Intracellular expression of β-galactosidase by the resulting virus vΔ35K/lacZ/35K^{Bcl-fr} was comparable to that of the deletion mutant vΔ35K/lacZ (Fig. 7); expression in SF21 cells was approximately 100-fold lower than that of 35K gene-containing viruses (wt/lacZ and vΔ35K/lacZ/35K^{+}) but was nearly equivalent to that of the two viruses in TN368 cells. The *BclI*-frameshift mutant also induced early cell lysis (12 to 24 h) in SF21 cultures that was indistinguishable from that of 35K deletion mutants (data not shown). Thus, generation of a frameshift mutation with minimal sequence alteration



Virus	β-galactosidase Activity (A_{420} /min/10^6 cells) TN368	SF21	SF/TN Activity (Percent of 35K+)
wt/lacZ	46.9 ± 2.0	4.7 ± 0.6	108
vΔ35K/lacZ/35K+	41.9 ± 6.3	3.9 ± 0.3	100
vΔ35K/lacZ/35KBcl-fs	98.0 ± 1.6	0.040	1.1
vΔ35K/lacZ/35KΔSpe	51.9 ± 2.7	0.065	1.3
vΔ35K/lacZ	29.7 ± 3.4	0.060	2.2

FIG. 7. Site-directed mutagenesis of p35. The wild-type (wt) 35K ORF (shaded arrow) encodes 299 amino acid residues (from ATG to TAA codons). Virus vΔ35K/lacZ/35K+ (35K+) contains the full-length ORF (shaded box) inserted immediately upstream from a polyadenylation signal (PA) at the polyhedrin locus (Fig. 1). The inserted 35K ORF of virus vΔ35K/lacZ/35KBcl-fs (35KBcl-fs) contains a 4-bp insertion at the unique *Bcl*I site, causing a frameshift that truncates p35 after 81 residues and fuses 16 out-of-frame residues (black box) to the C terminus; the hatched box depicts the remaining sequences of the ORF. The truncated 35K ORF (287 residues) of virus vΔ35K/lacZ/35KΔSpe (35KΔSpe) was generated by the introduction of a nonsense mutation at the *Spe*I site and removal of the 12 C-terminal amino acid residues as shown (7). Small vertical arrows mark the end of the 35K ORF for both mutants. Restriction site abbreviations are listed in Fig. 1. Intracellular β-galactosidase activity was determined 48 h after infection of SF21 or TN368 cultures, with an MOI of 1 PFU/cell. Values for β-galactosidase activity are the averages of duplicate infections. The activity ratio of SF21 to TN368 was calculated by normalizing the ratio of activity in SF21 and TN368 cells of the indicated viruses to that of vΔ35K/lacZ/35K+ (35K+), defined as 100%.

(4-bp insertion) was sufficient to cause the null phenotype and demonstrated that p35 is required for wild-type virus replication.

This conclusion was supported by the construction of a p35 truncation mutant in which the last 12 C-terminal residues were removed from the 35K ORF (Fig. 7). A nonsense codon was introduced at residue 288 by deleting the 145-bp region between the *Spe*I and *Eco*RI sites of the 35K gene fragment also inserted at the polyhedrin locus. Compared with other 35K mutants, virus vΔ35K/lacZ/35KΔSpe also directed synthesis of low levels of β-galactosidase in SF21 cells, but it directed wild-type levels in TN368 cells. vΔ35K/lacZ/35KΔSpe also induced premature lysis of SF21 cells. These results suggested that the C terminus, including the last 12 amino acid residues, is essential to p35 function or protein stability.

DISCUSSION

AcMNPV p35 null mutants exhibit cell line-specific growth restrictions. Our initial approach to identifying the function of genes encoded by the *Hin*dIII-K–*Eco*RI-S region of AcMNPV was to generate null mutants in which a specific gene was deleted (or replaced). Removal of various portions of the 35K ORF produced AcMNPV mutants that exhibited altered growth properties that were cell line specific. In cultured *S. frugiperda* (SF21) cells, production of BV by deletion mutant vΔ35K/lacZ was significantly reduced, ranging from 200 to 15,000 times less than that of wild-type virus, depending on the input MOI (Table 1). In contrast, yields of BV from 35K null mutants were indistinguishable from that of wild-type virus in cultured *T. ni* (TN368) cells. 35K null mutants also exhibited severely restricted levels of late gene expression in SF21 but not TN368 cultures. For example, expression of the *lacZ* gene under control of the very late polyhedrin promoter was 300-fold lower in SF21 cells infected with 35K null mutants than with wild-type virus (Fig. 3). Similar reductions in the synthesis of other late and very late proteins were detected in 35K null mutant-infected SF21 but not TN368 cells (Fig. 4).

By constructing a series of AcMNPV recombinants, including a frameshift mutant within the 35K ORF, we demonstrated that the growth restriction of the null mutants was due to loss of function of the 35K gene product (p35). Combined with the results of Clem et al. (3), these data provide strong evidence that the null phenotype is not due to loss of essential *cis*-acting DNA sequences (including regulatory or replication signals). This conclusion was supported by the finding that a functional 35K allele *trans* complemented 35K null mutants, as demonstrated by mixed infections in which wild-type virus reversed the mutant growth restrictions and stimulated BV production and very late gene expression (Fig. 5). Insertion of a 1.28-kb fragment containing the 35K ORF and its promoter at an alternative site (polyhedrin locus) restored late gene expression and BV production to wild-type levels in SF21 cells (Fig. 2 to 4). Thus, these sequences contained sufficient information (including *cis*-acting regulatory signals) to suppress the null phenotype. The finding that expression was independent of position within the virus genome, in addition to the dramatic growth advantage conferred by p35, has contributed to the development of the 35K gene as a useful selectable marker for in vitro isolation of recombinant viruses (21).

Role of p35 in AcMNPV replication. Clem et al. (3) recently demonstrated that 35K deletion mutants cause premature lysis of cultured SF21 cells that is the result of apoptosis (programmed cell death). This indicated that p35 is required to block the apoptotic process induced in response to infection by AcMNPV. Virus-induced apoptosis was distinguished by stereotypic degradation of intracellular DNA into oligonucleosome-sized fragments and premature cell lysis that involves progressive membrane blebbing. The reduced levels of 35K null mutant replication reported here may therefore be the combined effect of early cell lysis and other processes associated with apoptosis. Indeed, *trans*-complementation assays that used wild-type virus demonstrated a direct correlation between the suppression of early cell lysis and the levels of 35K null mutant replication (Fig. 5). In a way that is analogous to cytotoxic T-cell killing of virus-infected vertebrate cells in which apoptosis causes self-destruction of the target cell and prelytic fragmentation of virus DNA (23, 32), apoptosis in SF21 cells may also limit AcMNPV replication through direct fragmentation or inactivation of virus DNA. The nearly normal pattern of early protein synthesis followed by the severely restricted levels of late and very late proteins in 35K mutant-infected cells (Fig. 3 and 4) is consistent with loss of viral DNA, since late

gene expression depends on virus DNA replication (10, 30). The wild-type pattern of gene expression by these mutants in TN368 cells that are resistant to virus-induced apoptosis is also consistent with this possibility (Fig. 4). Nonetheless, it has not been ruled out that p35 directly regulates late gene expression or virus DNA replication in a cell line-specific manner.

Our results suggest that another function of p35 is to enhance the infectivity of BV. Compared with wild-type virus, significantly higher MOIs of 35K null mutants were required to induce cytopathic effects in SF21 cells. For example, when inoculated with a moderate MOI of vA35K/lacZ (5 PFU/cell), a large fraction of SF21 cells exhibited continued growth as indicated by increased host protein synthesis and lack of early lysis (Fig. 4). In contrast, an identical MOI of wild-type virus induced cytopathic effects and host protein shutoff in all cells. Consistent with a reduction in infectivity, increasing multiplicities of input virus produced a proportional increase in vA35K/lacZ progeny, whereas yields of wild-type virus remained relatively constant over at high multiplicities (Fig. 2A). 35K null mutants propagated in TN368 culture and subsequently assayed in SF21 cells exhibited similar properties, indicating that the defect was a direct consequence of the absence of p35 and not an indirect effect of the apoptotic response (i.e., fragmentation of packaged virus DNA) (18). Whether virus attachment, penetration, or uncoating is affected remains undetermined. The observation that 35K mutant replication was restored by complementation with wild-type virus, however, indicated that the lower infectivity was not solely responsible for reduced virus growth.

DNA viruses that counteract host antiviral defenses and prolong cell survival. The suppression of cell lysis and the resulting growth advantage conferred by p35 in tissue culture suggest that this protein functions to block virus-induced apoptosis within the host organism, thereby counteracting an insect antiviral defense (3). It has recently been shown that adenovirus encodes several proteins that function to evade host immunosurveillance by blocking the effects of apoptosis: gp19K blocks the transport of class I antigens of the major histocompatibility complex to the surface of adenovirus-infected cells, thereby preventing lysis by cytotoxic T-lymphocytes, while the 14.7K E3 and 19K E1B proteins protect virus-infected cells from apoptotic lysis by tumor necrosis factor (37, 38). Latent membrane protein 1 of Epstein-Barr virus also promotes survival of infected B lymphocytes by increasing expression of the host oncogene *bcl-2* (11, 13), which in turn suppresses apoptosis in these cells (15, 26). While the molecular mechanisms by which these proteins function remain undetermined, their importance to virus survival is suggested by the finding that both vertebrate and invertebrate pathogens have evolved strategies to counteract apoptosis.

An important question is whether p35 interacts directly with host components or whether it regulates other virus-encoded gene products that suppress the host response. Our studies indicate that the C-terminal 12 amino acid residues of p35 are critical for protein function or stability (Fig. 7). The C-terminal domain has a net positive charge due to a high proportion of lysine residues (7) and may therefore be important for interaction with other proteins or for targeting p35 to a specific location in the cell. Defining the interaction of p35 with other proteins as well as identifying essential protein domains should provide insight into the mechanisms by which this protein enhances AcMNPV growth and infectivity.

ACKNOWLEDGMENTS

We thank Robert Lerch for construction of virus vA35K/lacZ/35K*.

This work was supported in part by Public Health Service grant AI25557 from the National Institute of Allergy and Infectious Diseases and a University of Wisconsin Steenbock Career Development award (P.D.F.).

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